INVESTIGATION INTO THE UNIQUE ROLES OF MMP-2 AND MMP-9 IN TGFβ-INDUCED EPITHELIAL-MESENCHYMAL TRANSITION IN LENS EPITHELIAL CELLS

By

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ABSTRACT

Epithelial-mesenchymal transition (EMT) is a pathological process leading to the formation of anterior subcapsular cataract (ASC). Mediated by transforming growth factor beta (TGFβ), EMT involves the transformation of the monolayer of lens epithelial cells (LECs) into "spindle shaped" myofibroblasts, which manifest as plaques directly beneath the lens capsule. TGFβ-induced EMT leading to ASC has been associated with the upregulation of two specific matrix metalloproteinases (MMPs), MMP-2 and MMP-9. Having identified MMP-2 and MMP-9 as participants in the formation of cataracts, the specific roles of either of these MMPs have yet to be determined.

The current study utilized MMP-2 and -9 knockout (KO) mice to determine their unique roles in TGFβ-induced EMT. First, adenoviral injection of active TGFβ1 into the anterior chamber of MMP-2 KO mice led to the formation of distinct αSMA-positive anterior subcapsular plaques, in contrast to treated MMP-9 KO eyes, which were resistant. Additionally, an *ex vivo* mouse LEC explant system was established in these KO lines. In the isolated lens epithelial explants, TGFβ triggered a transformation of LECs from a tightly packed cuboidal monolayer to an elongated mesenchymal phenotype. This process involved a disruption in epithelial cell contacts indicated by a loss of E-cadherin, and an acquisition of myofibroblast marker, αSMA. In the absence of MMP-2, TGFβ was still able to induce EMT with E-cadherin loss and concurrent αSMA expression. In contrast, LEC explants from MMP-9 KO mice treated with TGFβ did not acquire a characteristic spindle-like phenotype and showed substantially less αSMA

expression. Results from both of these approaches were consistent; MMP-2, but not MMP-9, KO mice stimulated with TGFβ exhibited phenotypic changes typical of those described in ASC formation, namely a loss in cell attachments, multilayering of previously epithelial-like cells, and αSMA reactivity. Therefore, while MMP-2 is not necessary, MMP-9 is critical to TGFβ-induced EMT in LECs.

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αSMA	alpha smooth muscle actin
AdDL	adenovirus with no insert
AdLacZ	adenovirus with LacZ insert
AdTGFβ	adenovirus with TGβ1 insert
ASC	anterior subcapsular cataract
DAPI	4',6-diamidino-2-phenylindole
EMT	epithelial-mesenchymal transition
E-cadherin	epithelial cadherin
ECM	extracellular matrix
EMEM	Eagle's minimal essential medium
FBS	foetal bovine serum
FITC	fluorescein-isothiocynate
FHL 124	foetal human lens epithelial cells
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor

GTP	guanosine triphosphate
H&E	hematoxylin and eosin
IOL	intraocular lens
LAP	latency associated protein
LTBP	latent TGFβ binding protein
LEC	lens epithelial cell
MMP	matrix metalloproteinase
PBS	phosphate buffered saline
PCO	posterior capsule opacification
PCR	polymerase chain reaction
pfu	plaque forming units
ROCK	Rho-associated coiled-coil forming protein kinase
Tcf4/LEF	T-cell factor 4/ lymphocyte enhancer factor
TGFβ	transforming growth factor beta
TIMP	tissue inhibitor of matrix metalloproteinase

CHAPTER ONE

General Introduction

1.1 Cataracts

A cataract is an opacity that forms on the lens characterized by an increase in light scatter and a loss of lens transparency. The World Health Organization estimates that cataracts are responsible for 50% of all cases of blindness, accounting for 20 million blind individuals (Pascolini and Mariotti, 2011). Treatment involves surgical removal of the cataractous lens, replacing it with a synthetic intraocular lens (IOL). While surgery is an effective form of treatment, it is not readily available in developing countries, while patients in developed countries place a financial burden on the health care system. In the United States over 1.2 billion cataract operations are performed per year, carrying a cost of $3.4 billion (West, 2000). Beyond steep costs, surgery also brings about complications with 35% of patients forming secondary cataract or posterior capsule opacification (PCO) within two years following surgery, which may cause a secondary loss of vision (Apple et al, 1992). These statistics demonstrate a need for the development of novel therapeutic strategies that can help ease the "global burden of cataract" (Rao et al, 2011) that can be achieved through a deeper understanding of the underlying molecular mechanisms involved in cataract formation.

The lens (Figure 1) is a transparent, avascular tissue located in the anterior segment of the eye just behind the iris, which along with the cornea, transmits and focuses light onto the retina (Tholozan et al, 2007). The outermost layer of the lens, the lens capsule, is a thick basement membrane, which protects the lens from its outside environment. The fluid surrounding the capsule at the anterior region of the lens is aqueous humour, while the posterior lens is bathed in vitreous humour (Gordon-Thomson

et al, 1998). Immediately underneath the capsule lies a monolayer of cuboidal lens epithelial cells (LECs) found solely in the anterior portion of the lens, whose basal ends remain in contact with the capsule. Apart from the anterior region of the lens, the majority of the tissue is made up of long, thin fibre cells that span the entire length of the lens from posterior to anterior pole. In a normal adult lens, LECs differentiate into fibre cells beginning their proliferation in the germinative zone, a region found slightly anterior to the equator of the lens, and continuing their migration downwards to the transitional zone at the equator. It is here the LECs elongate and differentiate into fibre cells as they migrate inwards along the equatorial line (Tholozan et al, 2007). The most superficial fibre cells, found closest to the lens equator, are metabolically active, and nucleated. As the fibres progress deeper towards the lens nucleus, a process known as terminal differentiation occurs culminating in the loss of organelles, cytoskeletal elements and denucleation (Tardieu et al, 1992; Kenworthy et al, 1994; Michael and Bron, 2011).

Cataracts are classified based on age of onset and location within the lens. Congenital cataracts are visible at birth or soon after with mainly a genetic cause. Age-related cataracts can be nuclear, cortical or subcapsular. From birth the stiffness of the lens steadily increases until after 40 years of age the nucleus becomes denser than its surrounding cortex. A nuclear cataract is characterized by an abnormally dense fibre cell nucleus with increased light scatter and nuclear discoloration. They are formed in a steady manner and result in uniform opacities. In cortical cataracts, opacities form in the superficial, nucleated lens fibre cell layers and occur in a discontinuous manner resulting in asymmetrical opacities. UV radiation, diabetes, and corticosteroid intake are all

associated risk factors for cortical cataract (Michael and Bron, 2011). Subcapsular cataracts form directly beneath the lens capsule at either the anterior or posterior poles of the lens. The present work will focus on the mechanisms involved in anterior subcapsular cataract (ASC).

1.2 TGFβ Signaling in Lens Epithelial Cells

In pathological situations such as ocular trauma, ocular surgery or in association with certain diseases such as atopic dermatitis and retinitis pigmentosa (Lovicu and Robinson, 2004; Sasaki, 1998), LECs have the ability to differentiate and transform into "spindle shaped" cells, or myofibroblasts (Font and Brownstein, 1974; Novotny and Pau, 1984; Hay, 1995). This process is known as epithelial-mesenchymal transition (EMT; Figure 2) wherein the transformed LECs have lost their epithelial markers (E-cadherin, β-catenin) and concomitantly acquired mesenchymal markers (αSMA, N-cadherin, vimentin) (Powell et al, 1999; Kropp et al, 1999; Nieman et al, 1999), exhibiting atypical characteristics including reorganization of the actin cytoskeleton, multilayering, and migration (Miettinen et al, 1994; Hay, 1995; Moustakas and Heldin, 2007). This accumulation of abnormally proliferating epithelial turned mesenchymal cells manifests as plaques beneath the anterior lens capsule, leading to vision impairment due to the development of lens opacities, or ASC. The transdifferentiation of LECs is also typical of PCO following cataract surgery, where not all LECs are removed from the anterior capsule leading to the migration and growth of these remaining cells onto the previously cell-free posterior capsule (Apple et al, 1992). Therefore, understanding the mechanism

involved in EMT is critical to the prevention of both initial anterior subcapsular plaque formation, as well as the progression of secondary after-cataract impairment.

In pathologies where EMT is a hallmark, one specific growth factor, transforming growth factor β (TGFβ) has been identified as a primary mediator. TGFβ is one of many factors present in the aqueous humour of the anterior chamber that has been shown to regulate LEC activity, and doubles in concentration following cataract surgery (Cousins et al, 1991; Wallentin et al, 1998). It has been shown to play a key role in the transdifferentiation of epithelial cells of the lens into mesenchymal cells leading to cataract. The treatment of excised whole rat lenses *in vitro* with TGFβ disrupts the normal architecture of the lens and results in characteristic changes associated with the conversion of LECs into myofibroblasts. Additionally, treating *ex vivo* rat LEC explants with TGFβ triggers the formation of spindle-shaped cells immunoreactive for alpha smooth muscle actin (αSMA), a known marker of EMT (Gordon-Thomson, 1998). Furthermore, transgenic mice overexpressing TGFβ, specifically in the lens, have also been shown to develop ASC, mimicking human cataractogenesis (Srinivisan et al, 1998). Therefore, *in vitro* and *in vivo* induction of ASC through TGFβ overexpression in both mice and rats are practical models for the study of ASC in humans.

Three isoforms of TGFβ exist in mammals (-1, -2, -3), all of which are synthesized as dimeric precursor proproteins. Unusually, TGFβ is secreted in a latent complex associated through noncovalent interactions with its N-terminal latency-associated peptide (LAP). This latency complex also includes the latent TGFβ binding

protein (LTBP), which associates with LAP through disulfide bonds during secretion. Since the structure of LTBP is reminiscent of an extracellular matrix protein, it gets incorporated into the ECM, bringing with it the entire TGFβ latency complex. TGFβ activation occurs through proteolytic cleavage of the LTBP, which releases the TGFβ-LAP complex from the ECM, and exposes sites on the LAP allowing for cell surface interaction, previously masked by LTBP. A second round of proteolytic cleavage is necessary to release TGFβ from LAP, leaving un-bound TGFβ free to bind to its receptors and activate the TGFβ signaling pathway. Activation of the TGFβ complex can be induced through heat, extreme pH or molecules such as plasmin, αvβ6, and thrombospondin 1 (Lyons et al, 1990; Munger et al, 1997; Yu and Stamenkovic, 2000). In the eye, once TGFβ is activated it is regulated/inhibited by a number of biological molecules including α_2-macroglobulin, a serum protein found in the aqueous humour with a high affinity for active TGFβ, controlling its localization and preventing it from exerting its effects at inappropriate sites through clearance from extracellular fluids (Schulz et al, 1996; Munger et al, 1997).

1.3 Role of Matrix Metalloproteinases in EMT

TGFβ-induced EMT events have been associated with increases in a family of zinc-dependent endopeptidases, the matrix metalloproteinases (MMPs). MMPs have historically been seen as enzymes responsible for the degradation of the structural components of the extracellular matrix (ECM) necessary for maintaining normal physiological processes, such as regulating tissue remodelling during embryonic development and wound healing (Seomun et al, 2001). Recently, MMPs have also been

shown to cleave circulating cell surface molecules and other extracellular non-matrix proteins, contributing to the regulation of cell behaviour (Nelson et al, 2000; Sternlicht and Werb, 2001). Importantly, MMPs have been implicated in a number of ocular diseases including retinal disease, glaucoma, corneal disorder, scleritis, uveitis, pterygium (Sivak and Fini, 2002; Alapure et al, 2008) and accumulating evidence suggests MMPs play an integral part in the mechanism involved in cataract formation.

The MMP family consists of over 20 structurally related members that can be classified as membrane-bound (MMP-14, -15, -16, -17, -24, -25) or secreted proteins, which are further subdivided into four categories: collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -11, -12, -28), and matrilysins (MMP-7, -26) (Figure 3; Klein et al, 2011). The basic structural domains of MMPs are fairly conserved, each with a N-terminal signal peptide for secretion linked to a pro-peptide domain for sustaining enzyme latency. A catalytic domain with a zinc-binding region is attached to a hemopexin-like C-terminal domain functioning as a substrate recognition sequence, determining substrate specificity (Nagase, 1997; Murphy and Knauper, 1997; Sternlicht and Werb, 2001). Uniquely, the two gelatinases contain three cysteine-rich repeats within their catalytic region allowing for interactions with collagens and elastins (Bode et al, 1999; Murphy et al, 1994; Shipley et al, 1996).

MMPs are primarily regulated at the transcriptional level by growth factors, cytokines, cell-cell interactions, and their surrounding cellular environment. Most MMPs are constitutively secreted as latent enzymes, their pro-peptide domain containing an

unpaired cysteine residue that associates with the active zinc site within the catalytic domain. Their activity is localized near the cell surface and depends upon the balance between MMP activators and inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs), in the surrounding extracellular space (Brew et al, 2002).

Activation of the zymogen occurs through the proteolytic processing of the pro-domain, freeing the catalytic site for substrate cleavage (Van Wart and Birkedal-Hansen, 1990). The bulk of the secreted MMPs become activated extracellularly by various proteases, and by previously activated MMPs themselves. Alternatively, MT-MMPs (membrane-type MMPs), as well as secreted MMP-11 and -28, can be activated through intracellular pro-domain cleavage by furin, since these MMPs contain a specific amino acid sequence recognized by this proteinase (Pei and Weiss, 1995). Due to their intracellular activation and membrane-associated nature, MT-MMPs play a unique role in the activation, and cell-surface localization of their secreted family members. It has been well documented that the activation of latent MMP-2 occurs through the interplay between MT1-MMP, TIMP-2, and MMP-2, events taking place at the cell membrane that are unique to MMP-2 (Strongin et al, 1995). Following furin-mediated activation of MT1-MMP and its membrane localization, the N-terminal domain of TIMP-2 binds to the catalytic site of MT1-MMP (Vu and Werb, 2000). The C-terminal of TIMP-2 attracts the binding of proMMP-2, forming an MT1-MMP/TIMP-2/MMP-2 complex. A free, adjacent MT1-MMP, uninhibited by TIMP-2, cleaves the pro-domain of proMMP-2 thereby alleviating its latency (Deryugina et al, 2001; Sato and Takino, 2010).

Although TIMP-2 participates in the activation of MMP-2 in this case, the C-terminal domain acts as an MMP-2 inhibitor. This dichotomy of activator versus inhibitor depends upon the extracellular concentration of TIMP-2, with a low concentration promoting MMP-2 activation, while high levels lead to overall inhibition (Strongin et al, 1995; Butler et al, 1998; Sato and Takino, 2011). Maintaining a balanced level of TIMPs is vital for proper tissue organization, and any uncontrolled expression can lead to diseases such as cancer or fibrosis (Brew et al, 2000).

There are currently four TIMP isoforms identified (TIMP-1, -2, -3, -4), each with distinct affinities for different MMPs (Brew et al, 2000). As was mentioned in the previous paragraphs, TIMP-2 specifically forms a complex with proMMP-2 at the cell surface for zymogen activation. TIMP-1, on the other hand, forms a complex with proMMP-9, but the nature of this interaction is still unclear and unknown as to whether it amounts in MMP-9 activation (Goldberg et al, 1992).

Each MMP selectively degrades components of the ECM, leading to alterations in the surrounding microenvironment; however MMP-driven proteolysis does not end in mere ECM digestion and remodelling, but continues in the indirect regulation of cell behaviour and activity in an "outside-in" manner. The ECM regulates cell shape, growth, and motility. By extension, MMPs are also responsible for regulating these cell processes by acting on cell matrices. Through the disruption of matrix barriers MMPs can release and modify various growth factors and cytokines embedded within the ECM (Sivak et al, 2002). In this manner, MMPs are controlling the bioavailability and bioactivity of growth

factors and cytokines, and their receptors. These molecules are normally ECM-sequestered in an inactive state, and upon their release are free to activate signaling sequences, which are then propagated to the cell nucleus (Sivak et al, 2002; Kessenbrock et al, 2010). The gelatinases have been shown to release and activate TNFα, IL1β, and TGFβ from ECM constituents implicated in the pathways involved in angiogenesis, inflammation, and carcinogenesis (Yu and Stamenkovic, 2000; Sternlicht et al, 1999; Coussens and Werb, 2001; Tian et al, 2011).

These mechanisms of aberrant MMP activity are highly studied in tumorigenesis and various types of cancer systems, where cancer cells express high levels of MMPs and integrins correlating with a high degree of motility, EMT, and invasiveness (Bourboulia and Stetler-Stevenson, 2010). Tumour growth and metastasis are less prevalent in mice with specific MMP gene disruptions, indicating the involvement of MMPs in uncontrolled cell growth and invasiveness (Itoh et al, 1998). This can occur through MMP-driven substrate cleavage, which can produce fragmented proteins with novel biological activities when compared to the intact molecule, leading to intracellular rearrangement of the cytoskeleton, and consequent cell migration (Lochter et al, 1997; Noe et al, 2001; Bourboulia and Stetler-Stevenson, 2010). For example, MT1-MMP and MMP-2-induced cleavage of laminin-5 produces fragments, which through an exposed cryptic promigratory site stimulate cell migration in mammary epithelial cells (Gianelli et al, 1997; Koshikawa et al, 2000).

In the eye, MMPs have been implicated in corneal wound healing, proliferative retinopathies, and macular degenerations (Fini et al, 1995). In the lens, LECs are capable of synthesizing MMPs following lens injury by ultraviolet irradiation, oxidative stress, or cataract surgery (Alapure et al, 2008). MMPs are detected in the media of whole cataractous lenses compared to normal non-cataractous lenses, as well as capsular bags following sham cataract surgery, indicating a role for MMPs in a wound healing process in response to injury or stress (Tamiya et al, 2000). The most widely studied MMPs in the ocular tissue are MMP-2 and MMP-9. Richiert and Ireland (1999) demonstrated that expression of both gelatinases is induced in primary chick lens cells following, and specific to, treatment with TGFβ. Our lab has also demonstrated the enhanced secretion of both MMP-2 and -9 in the conditioned medium of whole rat lenses following TGFβ treatment. Furthermore, co-treatment of this *in vitro* lens cataract model with TGFβ and a specific MMP2/9 inhibitor suppressed anterior opacities that were otherwise visible on the lens when solely treated with TGFβ (Dwivedi et al, 2006), indicating a critical role for these two gelatinases in the formation of subcapsular plaques. This is thought to occur through the disruption of the adherens junction constituent E-cadherin, essential for proper formation and maintenance of epithelial cell junctions. A loss of E-cadherin, and thus a loss of intercellular contacts, correlates with EMT, and research is focusing on the proteolytic activity of MMPs to explain this correlation (Zheng et al, 2009). MMPs have been found to cleave the N-terminal extracellular domain of E-cadherin releasing a unique 72kDa fragment into conditioned medium of *in vitro* LECs, a phenomenon known as “E-cadherin shedding” (Dwivedi et al, 2006). Cleavage and downregulation of E-

cadherin by MMPs eliminates both the link between cells at the cell junction and the link between the cell's extracellular environment and its cytoskeleton. Losing cell-cell contacts has a drastic impact on the characteristics of the epithelial cells, such that changes in morphology and intracellular signaling events may lead to transcriptional activation of factors that promote an abnormal phenotype.

Having identified MMP-2 and MMP-9 as participants in the EMT process, the individual roles of either MMP in EMT have yet to be determined. Specifically focusing on cell contact dissolution and α-SMA induction will help to elucidate the mechanism involved in MMP-mediated TGFβ-induced ASC.

1.4 RhoGTPases and TGFβ-Induced EMT

The TGFβ signaling pathway also has an effect on the organization and regulation of the actin cytoskeleton, which has proven to be important for cell motility, differentiation and tissue organization (Figure 4; Moustakas and Heldin, 2008). Key regulators of the actin cytoskeleton belong to the Rho family of small GTPases (Bishop and Hall, 2000), which can be subdivided into three groups: Rho (RhoA, RhoB, RhoC), Rac (Rac1, Rac2), and Cdc42 (Vega and Ridley, 2008). The activation of Rho, Rac, and Cdc42 by extracellular ligands leads to the formation of stress fibres, lamellipodia, and filopodia, respectively (Hall, 1998). Since EMT involves reorganization of the actin cytoskeleton with the accumulation of focal adhesions and stress fibres, Rho is specifically thought to be a novel mediator of TGFβ-induced EMT in LECs (Kardassis et al, 2009).

Similar to other GTPases, Rho GTPases act as molecular switches cycling between an inactive, GDP-bound form, and an active GTP-bound form (Figure 5). Inactive Rho-GTPase is activated through guanine nucleotide exchange factors (GEFs), while active Rho-GTPase returns to its inactive state through intrinsic GTPase activity catalyzed by GTPase activating proteins (GAPs) (Van Aelst and D'Souza-Schorey, 1997). Interestingly, TGFβ is able to induce levels of activated GTP-bound Rho in a number of *in vitro* and *in vivo* animal systems (Masszi et al, 2003; Tavares et al, 2006; Lee et al, 2008), and a particular interaction between TGFβ and RhoA has been established in epithelial cells. The first study to demonstrate these findings was led by Bhowmick and colleagues (2001), who found a 4-fold accumulation of activated RhoA in mouse mammary epithelial cells after a 10 minute treatment with TGFβ when compared to control cells. In addition, they found that dominant-negative RhoA cells blocked the acquisition of a mesenchymal phenotype following TGFβ stimulation, however this mesenchymal transition was not blocked in dominant-negative Rac1 cells. A similar study by Cho and Yoo (2007) also found an increase in RhoA activation with TGFβ treatment in mouse lens epithelial cells. When the researchers inhibited the Rho effector Rho-associated coiled-coil forming protein kinase (ROCK) signaling pathway, TGFβ-induced EMT was abolished both in *in vitro* mouse LECs and *in vivo* mouse lenses. As a result, RhoA signaling appears to be required in order for TGFβ to be able to stimulate EMT.

1.5 Link Between RhoGTPases and MMPs

Additionally, a link between Rho-GTPase activation and MMP expression has been established in different cell types, furthering the hypothesis that Rho-dependent pathways are intertwined with TGFβ's induction of MMPs and subsequent EMT. For example, in a human monocytic cell line the inhibition of Rho proteins blocked the secretion of MMP-9, which was otherwise secreted in control cells upon stimulation (Wong et al, 2001), while in a fibrosarcoma cell line Rac1 was sufficient to induce the activation of MMP-2 (Zhuge and Xu, 2001). Since MMPs are involved in the degradation of extracellular matrix, and have specifically been shown to disrupt the E-cadherin/β-catenin pathway involved in cell-adhesion (Sternlicht et al, 1999), the involvement of Rho seems logical seeing as the activation of RhoA, in particular, has also been shown to be engaged in the disassembly of cadherin contacts between epithelial cells, migration and metastasis (Moustakas and Heldin, 2008). Collectively, these studies reveal that RhoA may be a very important link in determining how MMPs, specifically MMP-2 and -9, may contribute to the formation of ASC involving TGFβ-initiated EMT.

CHAPTER TWO

Main Hypothesis, Specific Aims and Rationale

2.1 Main Hypothesis

TGFβ is a central mediator of EMT in lens epithelial cells. As outlined above, evidence suggests that MMPs play a key role in orchestrating the EMT process, and subsequent ASC formation. Treating human LECs with TGFβ results in the specific induction of MMP-2 and -9 protein and mRNA. This combined with the fact that an MMP2/9 inhibitor abolishes the effects of TGFβ leads to the hypothesis that **both MMP2 and MMP-9 are essential components of TGFβ-induced EMT in LECs, exhibiting unique and cooperative roles.** Additionally, given that EMT involves the reorganization of the actin cytoskeleton, Rho-GTPases have also been implicated in EMT. It has been demonstrated that TGFβ activates RhoA, and that blocking the RhoA signaling pathway abolishes TGFβ-mediated EMT. Therefore, it is hypothesized that **TGFβ activates the RhoA signaling pathway in LECs leading to EMT.**

2.2 Specific Aims and Rationale

2.2.1 Aim 1

To examine the unique role of MMP-2 and -9 *in vivo* in mediating TGFβ-induced ASC

TGFβ1 will be overexpressed in the eyes of MMP-2 knockout mice using an adenoviral gene transfer method. This will determine the functional role MMP-2 plays in the formation of TGFβ-induced ASC *in vivo*. These results will be compared to previous adenovirus-mediated TGFβ overexpression studies in MMP-9 KO mice. These studies will determine if the presence of MMP-2 is critical for ASC formation.

2.2.2 Aim 2

Establish an *ex vivo* lens epithelium system and determine effect of TGFβ on explanted LECs

In addition, establishing a technique for *ex vivo* lens epithelium explants in mice will prove to be an effective model for the study of pathological conditions, such as ASC. Up until now, our lab has performed lens explants of rat eyes only due to their relatively larger sized lenses, however a technique for explanting mouse lens epithelia has not yet been established, nor has it been explicitly reviewed or documented in the literature. Therefore, my initial goal was to establish a mouse lens epithelium explant system. Once a proper procedure has been determined, lens epithelial explants from MMP-2 and MMP-9 KO mice will be treated with TGFβ, and subsequently stained for α-SMA, further clarifying the unique role of MMP-2 in LEC EMT.

2.2.3 Aim 3

To determine whether RhoA GTPase activation is necessary for TGFβ-mediated EMT in our cell line of human LECs.

Firstly, human lens epithelial cells will be treated with TGFβ and a Rho kinase (ROCK) inhibitor Y27632, subsequently staining for αSMA and F-actin to ensure that TGFβ is inducing EMT in these cells before progressing, and to characterize the effect of ROCK inhibition on the organization of the actin cytoskeleton in response to TGFβ. Following the induction of EMT in FHLs with TGFβ stimulation, levels of active GTP-bound RhoA

will be measured to determine if TGFβ exposure significantly changes activated Rho levels.

CHAPTER THREE

Materials and Methods

3.1 Aim 1: Investigation into the unique roles of MMP-2 and -9 *in vivo*

3.1.1 *Recombinant Adenovirus*

The individual role of MMP-2 was analyzed in ASC formation by overexpressing TGFβ1 in the eyes of MMP-2 knockout mice using a previously validated adenoviral gene transfer method (Margetts et al, 2005). Briefly, a recombination-deficient adenovirus containing the transgene encoding active human TGFβ1 (AdTGFβ1) was injected into the anterior chamber of 6-8 week old MMP-2 WT and KO mice (6), following a method previously used in our laboratory (Robertson et al, 2007). In order to produce constitutively and biologically active TGFβ1 protein, the TGFβ cDNA contained in the adenovirus includes two cysteine to serine point mutations at sites 223 and 225 ($AdTGF\beta^{223/225}$), located in the pro region of precursor TGFβ (Sime et al, 1997). As a negative control, an empty vector (AdDL) was also employed to ensure that the results are TGFβ specific.

3.1.2 *Adenoviral Injection of TGFβ1 in MMP-2 KO mice*

All animal studies were performed according to the Canadian Council on Animal Care Guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adenoviral vectors were intracamerally injected into MMP-2 knockout mice generated by the removal of exon 1 with a *pgk-neo* gene cassette (Itoh et al, 1997). MMP-2 WT and KO mice were anaesthetized with isofluorane and placed under a dissecting microscope. A volume of 5µl of $5x10^8$ pfu AdTGFβ or AdDL was injected

into the left eye of each mouse using a 33 gauge needle attached to a 10µl Hamilton syringe, while leaving the right eye a manipulation free negative control.

3.1.3 *Genotype Analysis*

DNA extraction and purification from mouse ear tissue was performed with a kit (DNeasy, Qiagen Inc.). Genotypes were determined by polymerase chain reaction (PCR) for the wild-type *Mmp2* allele (5'-CAACGATGGAGGCACGAGTG-3' and 5'-GCCGGGGAACTTGATCATGG-3'), *Mmp2* null allele (5'-GACCACCAAGCGAAACAT-3' and 5'-CAAGAAGGCGATAGAAGG-3'), wild-type *Mmp9* allele (5'-GTGGGACCATCATAACATCAC-3' and 5'-CTCGCGGCAAGTCTTCAGAGTA-3'), and *Mmp9* null allele (5'-CTGAATGAACTGCAGGCAGA-3' and 5'-ATACTTCTCGGCAGGAGCA-3'). All animals were housed under specific pathogen free conditions with food and water available ad libitum.

3.1.4 *Histology and Immunofluorescence*

Twelve days following injection the mice from different treatment groups were sacrificed and enucleated. Eyes were then processed overnight in 10% neutral buffered formalin, embedded in paraffin, and processed for routine histology. Four-micrometer thick midsagittal sections were stained with hematoxylin and eosin to examine the general morphology of the lens, specifically focusing on the monolayer of LECs. The tissue sections are further used for immunohistochemical detection using a monoclonal antibody against αSMA conjugated to FITC (1:100, Sigma) to detect for the presence of

EMT. All sections were mounted in Prolong Gold antifade reagent with DAPI (Invitrogen) for nucleus visualization.

3.2 Aim 2: EMT in *ex vivo* lens epithelium explants

3.2.1 *Ex Vivo Mouse Lens Epithelium Explants*

To obtain lens epithelial cell explants, 6- to 8-week-old MMP-2 and -9 WT and KO mouse lenses were placed in 35mm basement cell extract (BME)-coated culture plates (Trevigen) containing pre-warmed, serum-free medium 199 (M199) supplemented with 1% penicillin/streptomycin, 1% fungizone and 0.1% L-glutamine (Invitrogen). The lens is placed posterior-side up, the posterior pole is gently torn and the fiber mass slowly removed revealing the epithelium. Once separated, the epithelium is then pinned with a blunt tool to the bottom of the culture dish with the LECs directly bathed by the media and the outer lens capsule facing downwards.

3.2.2 *Histology and Immunofluorescence*

Confluent mouse epithelial explants cells were treated with serum-free M199 or TGFβ2 (R&D Systems) at 500pg/ml. After 48 hours, untreated and treated explants were fixed for 15 minutes with 10% neutral buffered formalin, washed in PBS and stained overnight at 4°C with a monoclonal antibody generated against αSMA-FITC conjugated (1:200, Sigma), and monoclonal anti-E-cadherin (1:200, BD Transduction Laboratories). Following antibody incubation, the explants were washed repeatedly with PBS and mounted in Prolong Gold antifade reagent with DAPI (Invitrogen).

3.2.3 *Statistical Analyses*

Levels of αSMA in stained explants were quantified using image quantification software (ImageJ, NIH, USA). Fluorescence was normalized to number of cells in each image and compared between groups expressed as the mean ± SEM. Data were tested for significance by Student's *t*-test (SPSS 19.0) and deemed significantly different when $p<0.05$.

3.3 Aim 3: RhoA GTPase activation in *in vitro* human lens epithelial cells

3.3.1 *FHL-124 Cell Culture*

Cells of the fetal human lens epithelial cell line, FHL 124 (Dr. Reddan), were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% FBS, 1% penicillin/streptomycin and 1% gentamycin. The cells were cultured in 8-well culture slides (BD Falcon) for immunostaining, 6-well plates for Western Blot, and 100 mm plates for RhoA measurement until they reached 60-70% confluence, at which point they were gradually serum starved in media containing 0.5% FBS for 24 hours, followed by complete serum starvation for another 24 hours to growth arrest the cells. Serum-starved cells were treated for 48 hours in preparation for immunofluorescence or 72 hours for Western Blot analysis with: (1) serum-free EMEM (control); (2) TGFβ2 (4ng/ml); (3) Y27632 (20μM); and (4) TGFβ (4ng/ml) + Y27632 (20μM). FHL cells used with the RhoA G-LISA were treated with TGFβ2 at 4 ng/ml for the following time points: (1) 48h; (2) 24h; (3) 4h; (4) 2h; (5) 1h; (6) 30m; (7) 12m; (8) 5m; and (9) 1m. At the end of each time point, the media was removed on ice, cells were washed with ice-cold PBS and

250µl cell lysis buffer (provided with the RhoA G-LISA assay) was added to each well for approximately 1 min. The lysed cells were scraped off each plate, centrifuged at 4°C, and subsequently frozen at -80°C.

3.3.2 *Histology and Immunofluorescence*

After 48hours the cells were fixed for 15 minutes with 10% neutral buffered formalin, washed in PBS and stained overnight at 4°C with a monoclonal antibody generated against αSMA-FITC conjugated (1:200, Sigma) and Alexa-Fluor-labelled phalloidin (1:200, Invitrogen). Following antibody incubation, the FHLs were washed repeatedly with PBS and mounted in Prolong Gold antifade reagent with DAPI (Invitrogen).

3.3.3 *RhoA Activation Assay*

Activated (GTP-bound) levels of RhoA were measured in the cell lysates treated with TGFβ at different time points to establish how soon RhoA activation occurred following TGFβ stimulation, and at what time point maximal RhoA activation was seen. This was carried out by using the RhoA G-LISA Activation Assay (Cytoskeleton, Inc.), according to the manufacturer's protocol. Briefly, the cell lysates are incubated in a 96 well plate, which is coated with a Rho-GTP-binding protein. Active, GTP-bound Rho in the lysate will bind to the wells, while inactive Rho is removed with a wash buffer. A specific RhoA antibody is then added to the wells, detecting bound active RhoA. The colorimetric signal is then read by measuring the absorbance at 490 nm using a microplate

spectrophotometer. Activated levels of RhoA are compared to non-activated cell lysate control levels.

3.3.4 *Western Blot Analysis*

To determine the presence of EMT, FHL 124 cell lysates were homogenized in Triton-X 100 lysis buffer containing protease inhibitor cocktail (Roche). A Bradford protein assay (Bradford, 1976) was performed to ensure that equal amounts of total protein were electrophoresed on a 10% SDS-polyacrylamide gel. The resolved bands were electro-transferred onto a nitrocellulose membrane. Membranes were blocked with 5% skimmed milk powder in Tris-buffered saline (50mM Tris base, NaCl pH 8.5) and 0.1% Tween-20 and then incubated overnight at 4°C with a monoclonal antibody against αSMA (1:8000, Sigma) or β-actin (1:8000, Abcam). Following this incubation, membranes were washed repeatedly in PBS, and probed with an HRP-conjugated secondary antibody (1:10000, Odyssey). Blots were visualized using the Li-Cor Odyssey imaging system and analyzed by image quantification software (ImageJ, NIH, USA).

CHAPTER FOUR

Results

4.1 Aim 1: Investigation into the unique roles of MMP-2 and -9 *in vivo*

4.1.1 *AdTGFβ injection leads to ASC plaque formation in absence of MMP-2*

The effects of adenovirally administered TGFβ1 (AdTGFβ) delivery into the anterior chamber (Figure 6) of MMP-2 WT and KO mouse lenses were first determined in order to assess whether MMP-2 was required for TGFβ-induced EMT. AdTGFβ injections were previously validated by Robertson et al (2007) through the injection of reporter constructs AdLacZ and AdGFP into the anterior chamber of mouse eyes. It was determined that transgene expression was detected in the lens epithelium four days post-injection in the absence of a capsular break, whereas transgene expression was absent in the lens epithelium of AdDL, empty vector, injected eyes. Therefore, administering TGFβ adenovirally is a reliable method for TGFβ overexpression in LECs. In the current study, histological sections of mice obtained 12 days following adenoviral injection were first stained for general cell architecture (H&E; Figure 7A, C, E) and then for αSMA reactivity (Figure 7B, D, F). This allowed us to visualize the arrangement of the LEC monolayer in response to TGFβ1, as well as verify that the anterior subcapsular plaques contain LECs that have transformed from epithelial cells to myofibroblasts.

Focusing on the anterior subcapsular monolayer of LECs, all WT mice (n=7) treated with AdTGFβ exhibited distinct ASC plaques consisting of multilayering of the epithelial cells (Figure 7A, black arrow). The cells within this multilayered plaque expressed αSMA (Figure 7B, white arrow), indicating myofibroblast transformation. These results

are in accordance with previous findings showing anterior subcapsular plaque formation with AdTGFβ delivery both 4 and 21 days post-injection (Robertson et al, 2007).

Having established that AdTGFβ delivery into the anterior chamber of the eye results in TGFβ-induced EMT in the LEC monolayer, the same technique was used in MMP-2 KO mice to see if the absence of MMP-2 affects plaque formation. Twelve days following AdTGFβ injection, all MMP-2 KO lenses (n=6) exhibited distinct ASC plaques consisting of focal multilayering of LECs in the anterior region (Figure 7C, black arrow) and that express αSMA (Figure 7D, white arrow). The ASC plaques in MMP-2 KO lenses resulting from TGFβ overexpression were comparable in size, location and frequency to their WT littermates, suggesting that MMP-2 is not critical for TGFβ-induced EMT and subsequent ASC plaque formation.

All of the mouse eyes injected with the AdDL, empty vector maintained a clear monolayer of epithelial cells along the anterior capsule (Figure 7E) with no detectable αSMA (Figure 7F) comparable to eyes left completely untreated. The fact that AdDL-injected sections did not display any uncharacteristic behaviour of LECs, namely LEC multilayering and αSMA reactivity, indicates that the injection of the adenoviral vector itself without TGFβ does not have any effects. This means that the effects of AdTGFβ delivery observed in our WT and MMP-2 KO mice can be attributed to the resultant overexpression of TGFβ.

AdTGFβ-injected eyes exhibited αSMA expression in the subset of LECs forming multilayered plaques in both MMP-2 WT and KO mouse eyes (Figure 7A and 7C, right),

demonstrative of EMT, whereas AdDL-injected or untreated control lenses produced no detectable αSMA in the LEC monolayer (Figure 7B and 7D, right).

Importantly, in all cases, the treated anterior lens capsule was not ruptured in any way and remained intact, protecting the lens epithelium from the contents of the anterior chamber and preserving it in its natural state. Beyond alterations in the lens epithelium, the delivery of TGFβ affected the architecture of the cornea (Figure 8). Untreated mouse eyes (Figure 8A) display a well-organized cornea with stromal cells neatly packed and aligned in parallel with the epithelial corneal cells. There is a clear separation between the lens capsule and the stroma through a continuous layer of the corneal endothelium. In contrast, the injection of AdTGFβ (Figure 8B) caused a clear disorganization and scatter of the epithelial and endothelial corneal cells. Stromal cells were no longer tightly packed in parallel, obtaining an elongated phenotype leading to a thickening of the corneal stroma. An absence of a continuous endothelium allowed for stromal cells to be in contact with the lens capsule meaning that the anterior chamber was no longer prominent. Changes in the corneal layers, which were not observed in AdDL-injected eyes (see Figure 7F), seem to be attributed to the overexpression of TGFβ, in line with previous AdTGFβ studies in our lab (Robertson et al, 2007) as well as TGFβ-transgenic mice presenting with these same corneal features (Srinivisan et al, 1998; Flugel-Koch et al, 2002).

4.2 Aim 2: EMT in *ex vivo* lens epithelium explants

4.2.1 Induction of EMT requires MMP-9, but not MMP-2

Next, we sought to validate our adenoviral studies and get a closer look at the LECs of our MMP KO mice. To achieve this, we utilized an *ex vivo* lens epithelium explant system. Once isolated, the LECs remain adhered to the lens capsule, the latter being in contact with the coated culture dish, while the LECs are bathed in medium. Confluent mouse lens epithelial cells when left untreated maintain a tightly packed, cuboidal, cobblestone-like appearance in a monolayer (Figure 9A). When these lens cells are treated with TGFβ their morphology drastically changes: they loose their cell-cell contacts, and begin to elongate into a spindle shape (Figure 9B).

In order to verify that these morphological changes in mouse LECs in response to TGFβ have undergone EMT lens explants were stained for E-cadherin (an epithelial marker) and αSMA (a myofibroblast marker). Importantly, untreated WT explants exhibited the characteristic epithelial cell phenotype with clear E-cadherin staining at the cellular junctions (Figure 10A) accompanied by little to no αSMA expression (Figure 10B). The transformation of LECs after TGFβ treatment leads to a decrease in E-cadherin at the cell periphery (Figure 10D) as well as distinct αSMA expression (Figure 10E). This increase in αSMA levels in TGFβ-treated LECs is significant (~7-fold) relative to untreated controls ($n=7$; $p<0.05$; Figure 10F), indicating that TGFβ has induced EMT in these *ex vivo* epithelial cells.

MMP-2 KO explanted cells when left untreated maintained a cuboidal arrangement with E-cadherin staining at the intact cell junctions (Figure 11A) and little to no αSMA expression (Figure 11B), similar to WT LECs. Notably, in the absence of MMP-2, TGFβ was able to induce a phenotypic transformation in the epithelial cell monolayer with a clear loss of E-cadherin at the cell junctions (Figure 11D) and a significant (~12-fold) increase in expression of filamentous αSMA (Figure 11E and G; $n=7$; $p<0.05$) comparable to treated WT explants. These data substantiate the results from the AdTGFβ injection experiments and strongly suggests that MMP-2 is not required for TGFβ-mediated EMT.

In contrast, MMP-9 KO lens epithelial explants when left untreated do not resemble WT untreated explants. In the absence of MMP-9 and without stimulation with TGFβ, a low level of E-cadherin is apparent (Figure 12A) and remains low upon TGFβ treatment (Figure 12D). In terms of myofibroblast marker acquisition, untreated MMP-9 KO LECs expressed little to no αSMA (Figure 12B). Importantly, in the absence of MMP-9, LECs treated with TGFβ did not acquire a characteristic spindle-like mesenchymal phenotype, and exhibited little to no αSMA expression (Figure 12E) that did not significantly differ from MMP-9 KO untreated controls ($n=5$; Figure 12G). A lack of significant TGFβ-induced αSMA expression in the absence of MMP-9 suggests a critical unique role for MMP-9 in TGFβ-induced EMT.

4.3 Aim 3: RhoA GTPase activation in *in vitro* human lens epithelial cells

4.3.1 *TGFβ treatment upregulates active RhoA levels*

First, the ability for TGFβ to increase levels of active RhoA in our FHL 124 cells was determined. RhoA activation, the GTP-bound form only, was measured using a RhoA activation assay (G-LISA) that emits a colorimetric signal specifically for the amount of activated RhoA in each sample. Levels of activated RhoA in response to TGFβ treatment were measured in FHL cell lysates at 1, 5, 12, 30 minutes, and 1, 2, 4, 24, 48 hours normalized to untreated, serum starved control cell levels (Figure 13). An immediate increase in RhoA activation after 1 minute of TGFβ treatment was seen with a significant upregulation occurring after 5 minutes of treatment to nearly 2 times that seen in control cells ($p<0.05$). Levels then slightly decay thereafter until significant upregulation is again seen with 1, 2, and 4 hour treatment periods, again to twice the amount of RhoA-GTP compared to untreated controls ($p<0.05$).

4.3.2 *Rho signaling pathway inhibition prevents TGFβ-mediated effects on LECs*

Since ROCK is a downstream effector of RhoA-GTPase (Leung et al, 1996) we investigated the role of RhoA signaling on TGFβ-induced EMT using a synthetic ROCK inhibitor (Y27632). FHL cells were treated with TGFβ (4ng/ml) in the presence and absence of Y27632 (20μM) and analyzed by immunolabeling and microscopy (Figure 14). After 72 hours, TGFβ treatment induced the expression of filamentous αSMA, compared to control untreated cells, which expressed minimal αSMA. This indicates that TGFβ leads to EMT in FHL cells. When co-treated with TGFβ and Y27632, TGFβ-

stimulated αSMA expression was almost entirely prevented, suggesting that the blockade of the Rho effector blocks TGFβ-induced EMT in FHL cells. Treatment with the ROCK inhibitor Y27632 alone did not induce αSMA expression.

To further this data, αSMA levels were quantified by Western analysis in FHL cell lysates under the same treatment conditions. Following 72 hours of TGFβ treatment, αSMA expression was induced to nearly seven times the level seen in untreated control cells ($p<0.05$; Figure 15). When TGFβ was coupled with Y27632 inhibitor the αSMA expression remained at the basal level not significantly different from untreated control cells (Figure 15). These results indicate that the inhibition of RhoA/ROCK signaling prevented TGFβ-induced αSMA. This suggests that RhoA activation is required in order for TGFβ to induce αSMA expression in LECs.

Microscopic examination of F-actin by staining with phalloidin in these same cells revealed that TGFβ treatment stimulated the dissolution of cell-cell contacts, and rearrangement of the actin cytoskeleton to a spindle morphology leading to the formation of actin stress fibres. The actin cytoskeleton in untreated and Y27632 treated controls possessed a more cortical arrangement of cell-cell junctions with a less pronounced formation of stress fibres when compared to TGFβ treated cells; however these control cells were not completely cuboidal: some had lost their cell-cell contacts, and stress fibre formation, while not as extensive as in the presence of TGFβ, was still evident. When cells were treated with TGFβ in the presence of Y27632, the amount of stress fibre formation seen with TGFβ treatment alone was diminished entirely, indicating that

TGFβ-induced stress fibre formation is dependent on Rho activation and signaling. Taken together, these data underline the requirement of RhoA/ROCK signaling in TGFβ-mediated EMT.

CHAPTER FIVE

Discussion

The objective of the present study served to delineate and distinguish between the roles of MMP-2 and MMP-9 in the transdifferentiation of LECs in EMT. Having established that both of these gelatinases are required for TGFβ-mediated EMT in various cell types, including LECs, their individual roles remained unknown. The current work has demonstrated that in the absence of MMP-2 TGFβ is still able to exert its EMT-inducing effects on the lens epithelium, whereas the absence of MMP-9 prevents these effects. These results were demonstrated both in an *in vivo* model using adenovirally-administered TGFβ and in an *ex vivo* lens explant system, all of which advantageously utilized MMP-2 and MMP-9 KO mouse lines. Results from both of these approaches were consistent; MMP-2, but not MMP-9, KO mice stimulated with TGFβ exhibited phenotypic changes typical of those described in ASC formation, namely a loss in cell attachments, multilayering of previously epithelial-like cells, and αSMA reactivity.

Recent work in our laboratory also examined the effect of AdTGFβ injections in MMP-9 KO mice (Dwivedi et al, 2006b). The MMP-9 mouse eyes were injected with AdTGFβ in the same manner as the MMP-2 KO mice. All MMP-9 WT mouse eyes injected with AdTGFβ at 4 (n=7), and 21 (n=2) days post-injection exhibited focal multilayering and αSMA immunoreactivity. However, only one of the MMP-9 KO eyes exhibited these characteristics (day 4 (n=5); day 12 (n=4); day 21 (n=7)), while all others injected did not exhibit ASC formation. Thus, unlike MMP-2 KO mice which exhibited ASC after AdTGFβ, MMP-9 KOs were resistant to ASC formation. These results suggest that MMP-2 is not necessary for the EMT of LECs in anterior subcapsular cataract formation *in vivo,* whereas MMP-9 is. Thus, MMP-2 does not play a unique role in

mediating TGFβ-induced EMT and points to MMP-9 as a critical component associated with ASC formation.

The notion that MMP-9 is the specific gelatinase required in order for EMT to progress is furthered through studies in our laboratory utilizing a transgenic mouse model overexpressing self-activating TGFβ1. These mice exhibit ASC fibrotic plaque formation beginning three weeks after birth, yet when crossed with the MMP-9 KO line the presence of multilayered plaques was abolished in 75% of the TGFβ-overexpressors lacking MMP-9, with no visible αSMA immunoreactivity or collagen deposition (Pino et al, 2008). Ridding of MMP-9 seems to have a protective effect on the lens epithelium from a TGFβ-induced fibrotic response, specifically implicating MMP-9 in the process.

These results are in line with our current findings in the anterior lens epithelium explants using MMP-2 and -9 KO mice providing further evidence of the critical role of MMP-9. Explanted LECs when left untreated maintained a tightly knit, cobblestone arrangement expressing no αSMA. TGFβ treatment leads to cell-cell detachment with a loss in E-cadherin, an elongation in cell shape, and expression of αSMA. These same changes in response to TGFβ are seen in MMP-2 KO explants, yet treated MMP-9 KO explants fail to react in this manner and instead preserve their native, untreated state. In the absence of MMP-9 the LECs stimulated with exogenous TGFβ are indistinguishable from untreated explants, supporting the claim that TGFβ's induction of EMT is mediated through MMP-9.

MMPs are generally not expressed or secreted in lens cells unless they are stimulated by growth factors, injury/stress or cataract surgery. For example, MMP-2 and -9 levels were induced in response to TGFβ in cultures of chick lens cells, human capsular bags, human LEC lines, as well as whole rat lenses (Richiert and Ireland, 1999; Wormstone et al, 2002; Seomun et al, 2001; Dwivedi et al, 2006a). This MMP induction spurred on by TGFβ is abolished if co-treated with either the broad-spectrum MMP inhibitor GM6001 (Ilomastat) or an MMP-2/9 specific inhibitor (Dwivedi et al, 2006a). MMPs are well-established mediators of the proteolytic processing of the ECM, which leads to growth factor and cytokine release as well as a disruption of cell-ECM and cell-cell contacts. The latter involves the cleavage of the extracellular domain of E-cadherin, and dissociation of the E-cadherin/β-catenin complex, amounting in free cytosolic β-catenin that is then able to translocate into the nucleus and activate proliferation. A study by Ho et al (2001) determined that MMP inhibition was able to regulate fibroblast cell adhesion and this was dependent on tampering with the normal stoichiometric ratio of 1:1 between MMPs and endogeneous inhibitors of MMPs, TIMPs. The downregulation of TIMP-1, tipping the MMP:TIMP axis in favour of MMP activity, led to reduced contact inhibition and multilayering of cells. These characteristics were coupled with a reduction in cadherin levels, involved in cell-cell adhesion, and a disruption in the assembly of focal adhesion complexes, involved in the formation of cell-ECM contacts. Adding recombinant TIMP-1 or an MMP inhibitor restores fibroblast adhesion and aggregation through the stabilization of cadherin-based adhesion at cell junctions and the re-establishment of cell-ECM adhesion evident through co-localization of vinculin and

$p125^{FAK}$ at points of focal contact (Ho et al, 2001). The dissolution of intercellular contacts, through the loss of E-cadherin, is widely recognized as an early step in the EMT process.

In *ex vivo* whole rat lenses, a 72-kDa E-cadherin fragment, compared to the 120-kDa intact molecule, appears following TGFβ stimulation concurrent with the induction of secreted MMP-2 and -9. TGFβ treatment also leads to a decrease in E-cadherin mRNA in the subcapsular plaques of rat lenses. Again, GM6001 or the MMP-2/9 specific inhibitor attenuated the shedding of E-cadherin and rescued the decrease in mRNA levels in this system. This suggested that MMP-2 and/or -9 is specifically involved in post-translational E-cadherin processing. The MMP-dependent E-cadherin ectodomain shedding phenomenon is not only specific to TGFβ, but is also seen in nitric oxide-treated murine colonic epithelial cells, where the nitric oxide-induced E-cadherin cleavage product and subsequent increase in cytosolic β-catenin are reversed upon MMP inhibition (Mei et al, 2002).

The present work in explanted mouse LECs corroborates evidence that extracellular proteolysis influences the adhesive properties of cells (Zheng et al, 2009; Hay and Zuk, 1995; Auersperg et al, 1999; Vieminckx et al, 1991). As expected, TGFβ treatment of explanted LECs led to a visible loss in E-cadherin at the cellular junctions. This decrease in E-cadherin in response to stimulation was also evident in the absence of MMP-2, indicating that MMP-2 itself does not impact TGFβ-induced E-cadherin loss, and may not be required for E-cadherin fragmentation. Interestingly, in the absence of

any TGFβ stimulation, MMP-9 KO LECs already had significantly lower levels of E-cadherin at cellular junctions when compared to untreated WT or MMP-2 KO LECs. Since E-cadherin levels were already significantly lower in untreated MMP-9 KO mouse LECs, it is difficult to definitively assess whether TGFβ led to a further loss in E-cadherin; however, from a visual standpoint E-cadherin levels seem to remain relatively unchanged between untreated and treated LECs in the absence of MMP-9.

The de-stabilization of cell adhesion in the lens cell explants in response to solely a lack of MMP-9 did not coincide with αSMA expression. A disruption of E-cadherin alone, an early hallmark of EMT, has recently been shown to lead to αSMA expression (Zheng et al, 2009), yet without the presence of MMP-9 a loss of E-cadherin is not sufficient to induce the transformation to a myofibroblastic phenotype. This underlines the importance of MMP-9 in EMT both in the initial stages of E-cadherin-mediated cell-cell contact, as well as further downstream in the acquisition of characteristics of myofibroblasts. Although the loss of E-cadherin has been attributed to cleavage by MMP-9 in other studies (see below), this is the first time MMP-9 has been linked with E-cadherin in a non-pathological situation. Importantly, MMP-9 mRNA has been found to be constitutively expressed in the lens, in contrast with MMP-2, which is induced in the lens following treatment with growth factors or during cataract formation (Reponen et al, 1992). MMP-9 promoter activity has also been found to be constitutively active in the lens epithelium in an *in vivo* transgenic mouse (Mohan et al, 1998). The differential pattern of expression between these two closely related gelatinases implies that they are controlled separately at the transcriptional level. It is possible that basal levels of MMP-9

play a part in the synthesis, stabilization or maintenance of cell contacts. This can occur in either a direct or indirect manner through the release or activation of other factors from the ECM that may regulate normal E-cadherin. In the future, it would be beneficial to add recombinant MMP-9 to MMP-9 KO lens explants to determine whether this could rescue E-cadherin at the cell junctions in untreated samples, as well as mediate TGFβ-induced E-cadherin cleavage.

Recent studies have emerged focusing on the link between MMPs, E-cadherin and consequent EMT in other systems. Zheng et al (2009) demonstrated that MMP-mediated disruptions in E-cadherin triggered EMT, events that were downstream of TGFβ. This study showed that E-cadherin loss was MMP-dependent and stressed that the dissolution of cadherin contacts is not just a consequence of TGFβ-induced EMT, but a cause of it. E-cadherin disassembly is thought to cause EMT through nuclear translocation of β-catenin, which activates the downstream transcriptional factor Slug, which induces EMT and further represses E-cadherin transcription (Zheng et al, 2009). From the reverse point of view, forced expression of E-cadherin alone in mesenchymal cells or transformed tumour cells was shown to lead to mesenchymal-epithelial transition, and suppress invasiveness (Hay and Zuk, 1995; Auersperg et al, 1999; Vieminckx et al, 1991). More specifically, Symowicz et al (2007) used ovarian epithelial carcinomas to demonstrate that E-cadherin shedding resulting from the progression of EMT was directly dependent on the protease activity of MMP-9, where blockade of MMP-9 function inhibited the presence of E-cadherin fragments. Previous studies in our lab have also implicated MMP-9 as the primary mediator of E-cadherin cleavage, showing that rat LEC explants treated

with recombinant MMP-9 alone facilitated the loss of full length E-cadherin and β-catenin at the cell periphery, similar to that observed following treatment with TGFβ. In this same study, co-treating explants with TGFβ and an MMP-9 specific inhibitor was able to prevent TGFβ-mediated E-cadherin and β-catenin downregulation (unpublished data). Therefore, based on our current results together with previous work in other systems, MMP-9 is a key mediator of E-cadherin in the lens epithelium.

TGFβ-induced αSMA expression was unaffected by the absence of MMP-2, which begs the question why MMP-2 is induced in response to TGFβ, and in what aspect of EMT it is involved. One possibility could be that MMP-9 is upstream of MMP-2. Regarding the temporal expression pattern of MMPs, studies by Nathu et al (2009) determined that TGFβ-induced MMP-9 mRNA levels preceded that of MMP-2 mRNA by two days in whole rat lens culture. MMP-9 mRNA induction also preceded that of MT1-MMP mRNA elevation, which coincided with the induction of MMP-2 mRNA. Since MT1-MMP is a known activator of MMP-2, MMP-9 appears to precede the induction of not only MMP-2, but also its regulatory factors. Further studies in human LECs determined that treatment with recombinant MMP-9 led to an increase in MMP-2 protein, correlating with αSMA expression (Nathu et al, 2009). Earlier studies in other systems also provide evidence that MMP-9 works upstream of MMP-2. For example, in corneal wound healing MMP-9 is known to be involved in the initial stages of repair, such as corneal re-epithelialization, whereas MMP-2 is involved in later stages of matrix degradation (Mohan et al, 2002). In addition, studies in carotid arteries found a significant early elevation in MMP-9 following injury-induced remodeling, preceding

MMP-2 and its downstream role in artery shrinkage (Godin et al, 2000). These results (our own included) provide a potential role for MMP-9 as an inducer of MMP-2 and may explain why, in the absence of MMP-2, LECs still undergo a loss of E-cadherin and expression of αSMA. In our case, MMP-2 function is not required for TGFβ-induced EMT to progress both *in vivo* and *ex vivo*, however this does not discount the possibility that it participates in a redundant fashion.

MMP-9, acting upstream of MMP-2, may modulate MMP-2 levels through its ability to release and activate growth factors and could explain why MMP-9 is critical for TGFβ-induced EMT. Yu and Stamenkovic (2000) reported on a novel mechanism for TGFβ activation involving the proteolytic cleavage and release of TGFβ from its latency complex by MMP-9. In order for this to occur, CD44 is required to recruit MMP-9 to the cell surface, concentrating it near potential substrates, such as latent TGFβ, pro-MMP-2, or pro-MMP-9 itself (Nagase, 1997). In this manner, the early induction of MMP-9 may serve to stimulate further TGFβ activity and signaling, which will in turn stimulate further MMP activation, drastically and efficiently altering the makeup of the local microenvironment and causing a pathological situation such as EMT. Another potential way MMP-9 may induce MMP-2 could be through the dissociation of β-catenin from E-cadherin. Upon translocating to the nucleus, β-catenin complexes with the Tcf4/LEF transcription factor. Takashi et al (2002) found a Tcf4 binding sequence in the MT1-MMP gene, and demonstrated that this motif directly binds with the β-catenin/Tcf4 complex affecting its promoter activity. Having established that MT1-MMP is the most common physiological activator of MMP-2 (Sato and Takino, 2010) provides another

possible mechanism for MMP-2 activation by MMP-9 through E-cadherin/β-catenin dissociation.

The cytoplasmic domain of E-cadherin interacts with the actin cytoskeleton via α-, β-, and p120 catenins, making any changes at the cellular junctions affect the reorganization of the cytoskeleton (Yap et al, 1997). This provides a link between E-cadherin and Rho, the main mediator of the actin cytoskeleton. The assembly and establishment of cadherins at adherens junctions lead to a decrease in RhoA activity (Noren et al, 2001); however, a low level of RhoA is necessary for proper E-cadherin assembly (Zhong et al, 1997; Jou and Nelson, 1998). This supports the view of cadherins, and their role in cell adhesion, not merely as structural components, but as active signaling molecules. As is the case in other systems, the current study showed a rapid increase in RhoA activity (GTP-bound form) in response to TGFβ in cultured human LECs. Immunofluorescence images of FHL cells show stress fibre formation and αSMA reactivity following TGFβ treatment, while co-treatment with TGFβ and the ROCK inhibitor abolished any TGFβ-mediated changes. These results are in line with similar studies documenting TGFβ-mediated activation of RhoA corresponding with the formation of actin stress fibres, the effects of which are nullified through inhibition of the downstream Rho pathway (Bhowmick et al, 2001; Cho and Yoo, 2007). The fact that maximal RhoA activity occurs within 5 minutes of TGFβ treatment indicates that it is one of the most upstream elements in the TGFβ signaling pathway. Since RhoA-regulated actin reorganization affects E-cadherin at the cell junctions, it is possible that the disruption of intercellular contacts in response to TGFβ occurs through RhoA/ROCK

signaling. Seeing as MMPs are responsible for E-cadherin cleavage, it is of interest to determine whether MMP expression, MMP-9 in particular, is downstream of RhoA and contributes to the early E-cadherin loss leading to EMT. The finding that low levels of RhoA are needed in normal epithelial cells for proper assembly and maintenance of adherens junctions, while high levels of RhoA are disruptive and lead to a fibroblastic phenotype (Zhong et al, 1997; Jou and Nelson, 1998) seems to coincide with our data demonstrating a need for MMP-9 in proper E-cadherin assembly, while overexpression leads to E-cadherin loss and EMT. The similarity between these observations lends credence to the idea that MMP-mediated E-cadherin degradation in EMT is RhoA-dependent and provides a promising avenue to explore in the future.

Overall, the results of the current study in combination with emerging evidence in the field of EMT have led us to propose a mechanism for TGFβ-induced EMT in LECs, leading to ASC formation (Figure 16). Upon binding of TGFβ to its receptor, the activation of RhoA and its downstream targets is the earliest event, leading to F-actin polymerization and regulation of the actin cytoskeleton. This occurs through the hypothetical induction of the proteolytic activity of MMP-9 by RhoA, either through the increase in its transcriptional activation, or, through an increase in its secretion. Recently, Popova et al (2010) found that fibroblast differentiation into myofibroblasts was induced in a TGFβ/RhoA/ROCK-dependent manner, involving an increase in the secretion of MMP-9 with the ratio of active MMP-9 to its precursor remaining unchanged. Future studies will focus on the connection between RhoA signaling and MMP-9. Further downstream in our mechanism, the extracellular localization of MMP-9 to the cell

membrane leads to the proteolytic processing of the ectodomain of E-cadherin, or E-cadherin shedding. This facilitates the dissociation of β-catenin from the cytoplasmic region of E-cadherin, allowing β-catenin to translocate into the nucleus. There, it forms a complex with the Tcf4/LEF transcription factor, which, as mentioned above, has been found to be a direct target of the MT1-MMP gene (Takashi et al, 2002). An increase in MT1-MMP leads to the activation of MMP-2, allowing for it to act on its substrates. In addition to E-cadherin cleavage, MMP-9 may also directly act on the TGFβ/LAP/LTBP complex embedded in the ECM, releasing and activating latent TGFβ (Yu and Stamenkovic, 2000). Therefore, TGFβ signaling in this proposed mechanism proceeds through the RhoA/ROCK pathway, stimulating MMP-9 activity upstream of MMP-2, where MMP-9 cleaves E-cadherin and releases latent TGFβ, exacerbating TGFβ signaling through a positive feedback loop.

Isolating and explanting of the lens epithelium is an effective *ex vivo* model that is well suited for studying the mechanisms behind the transformation of these epithelial cells into a mesenchymal phenotype. Thus far, our laboratory has successfully and routinely utilized a method for lens explants of the postnatal rat eye, however a method for explanting the mouse lens epithelium had not yet been determined, the bulk of the issue being the relatively smaller size of the mouse lens. This system maintains the lens epithelium attached to its native basement membrane, the lens capsule, and separates it from the fiber cells of the remainder of the lens, allowing for easy treatment and microscopic visualization of LECs. It is particularly interesting to note that in contrast to the majority of cell types, lens cells are able to survive and sustain their phenotype

without the meddling of exogenous stimulants, such as serum, as long as they are in contact with their native matrix. Studies have demonstrated that isolated human capsular bags can be maintained in culture for over a year in protein-free medium and are capable of *de novo* protein synthesis throughout this period through an autocrine mechanism (Wormstone et al, 2001). This indicates the appropriateness of the lens explant model for use in these studies, mimicking the particular reaction of LECs to TGFβ stimulation from stressors, such as cataract surgery, in a contained and isolated manner. The drawback to the mouse LEC explant model is the low number of cells isolated from the small lens epithelium, not allowing for quantification of protein levels in the lysates nor in the conditioned medium, even when pooling multiple explants. Recent work in our laboratory has, however, demonstrated that mRNA levels can be accurately measured from a single mouse explant and provides an exciting alternative. Therefore, quantifying MMP-2 and -9 mRNA levels in mouse LEC explants following TGFβ treatment in the presence and absence of the RhoA/ROCK inhibitor will undoubtedly add to the current study, and compliment our qualitative explant data, while furthering our understanding of how TGFβ induces MMP-2 and -9.

The majority of published findings in the area of MMPs and EMT focus on MMP-2 and -9, however have not characterized their individual roles. This thesis has shown, for the first time, that MMP-9, but not MMP-2, is critical to TGFβ-mediated EMT in LECs. The exact manner in which MMP-9 is involved in EMT, and specifically the earlier stages of E-cadherin shedding, will be indispensible in unraveling the complexities of TGFβ-mediated EMT and provide insight into the pathogenesis of ASC formation, as

well as PCO development following cataract surgery. An accurate mechanistic pathway distinguishing between the roles and interactions of MMP-2 and -9 will be key in the development of therapeutic strategies for these ocular pathologies.

FIGURES

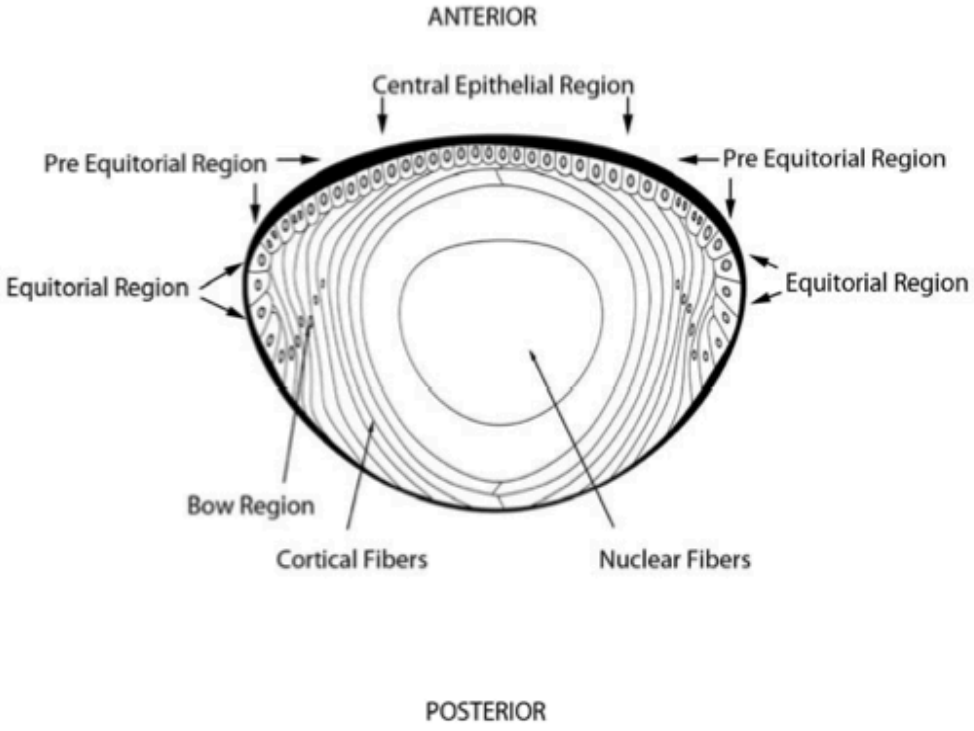


Figure 1. Cross section of lens. The lens capsule (thick black) surrounds the entire lens. The anterior lens is divided into three distinct regions. The central lens epithelial region is defined as the region where the LECs are quiescent. LECs at the pre-equatorial region are non-quiescent and are responsible for the generation of new lens fiber cells. LECs elongate at the equatorial region and subsequently undergo programmed nuclear and organelle loss as they become cortical fibers. The nuclear fibers at the centre of the lens are the primary lens fiber cells created at birth.

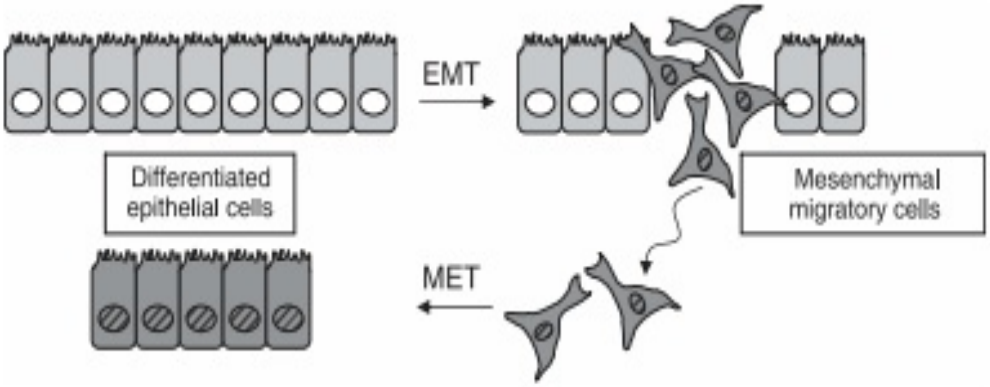
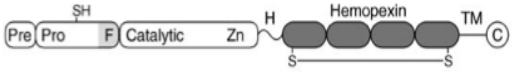
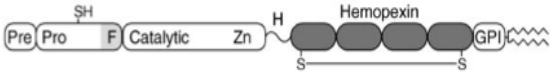


Figure 2. Epithelial-mesenchymal transition (EMT). The monolayer of epithelial cells transforms into migratory myofibroblasts, displaying fibroblast-like characteristics and expressing mesenchymal markers (Adapted from Moustakas and Heldin, 2007).

Membrane-associated

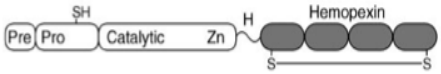


MMP-14
MMP-15
MMP-16
MMP-24



MMP-17
MMP-25

Collagenases and Stromelysins



MMP-1
MMP-8

MMP-3
MMP-10
MMP-11
MMP-12
MMP-28

Matrilysins

MMP-7
MMP-26

Gelatinases

MMP-2
MMP-9

Figure 3. Structure and classification of matrix metalloproteinases. MMPs are classified membrane-associated or secreted (collagenases, stromelysins, matrilysins, gelatinases) proteins. In general, MMPs have: 1) N-terminal signal peptide targeting the zymogen for secretion; 2) a propeptide domain maintaining latency; 3) zinc catalytic domain; 4) C-terminal determining substrate specificity.

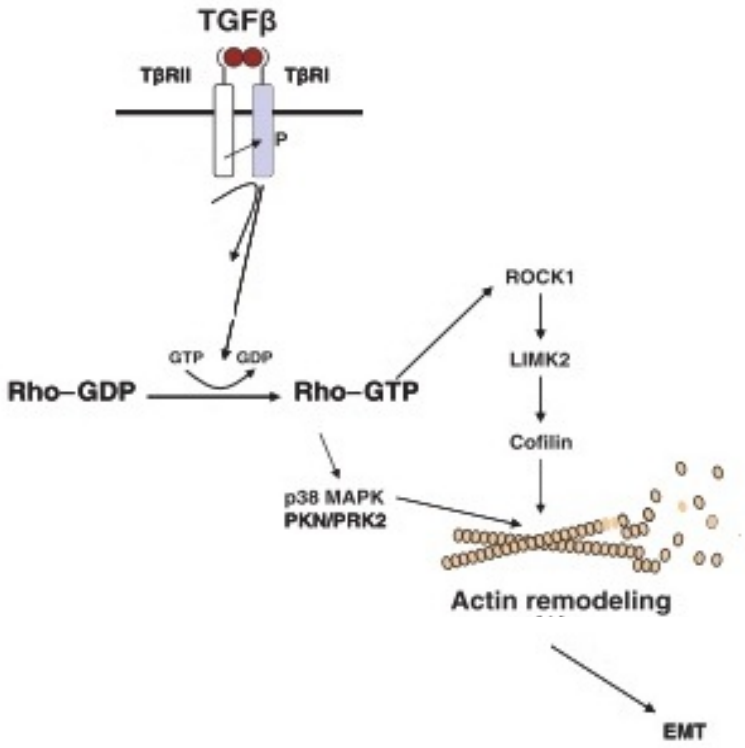


Figure 4. The reorganization of the actin cytoskeleton mediated by TGFβ signaling. Activation of Rho by the TGFβ signaling pathway leads to actin polymerization via the ROCK1/LIMK2/cofilin pathway, which in turn facilitates EMT (Adapted from Kardassis et al, 2009).

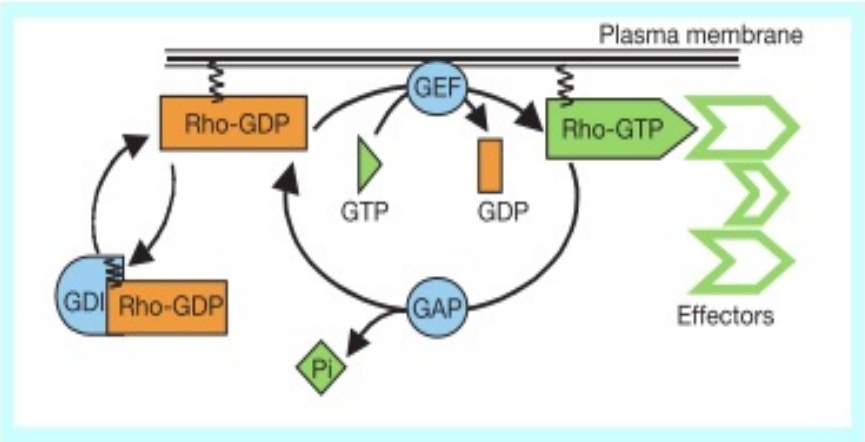


Figure 5. The cycling of Rho GTPases. RhoGTPases are active in their GTP-bound form, which is regulated by guanine nucleotide exchange facors (GEFs), and return to their inactive, GDP-bound form through intrinsic GTPase activity catalyzed by GTPase activating proteins (GAPs) (Adapted from Etienne-Manneville and Hall, 2002).

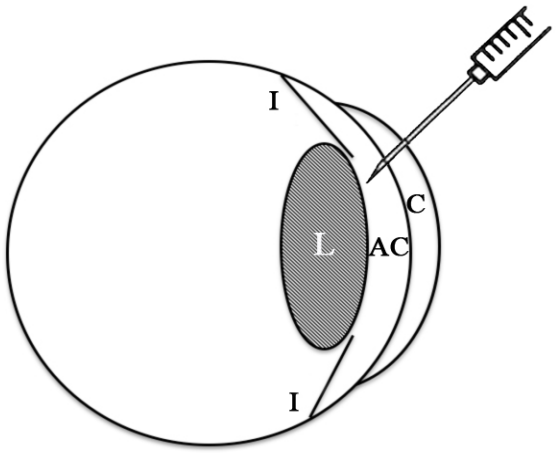


Figure 6. Intracameral injection of adenovirus. AdTGFβ was injected into the anterior chamber of MMP2 KO or WT mouse eyes, making sure not to puncture the lens. *AC*, anterior chamber. *C*, cornea. *I*, iris. *L,* lens.

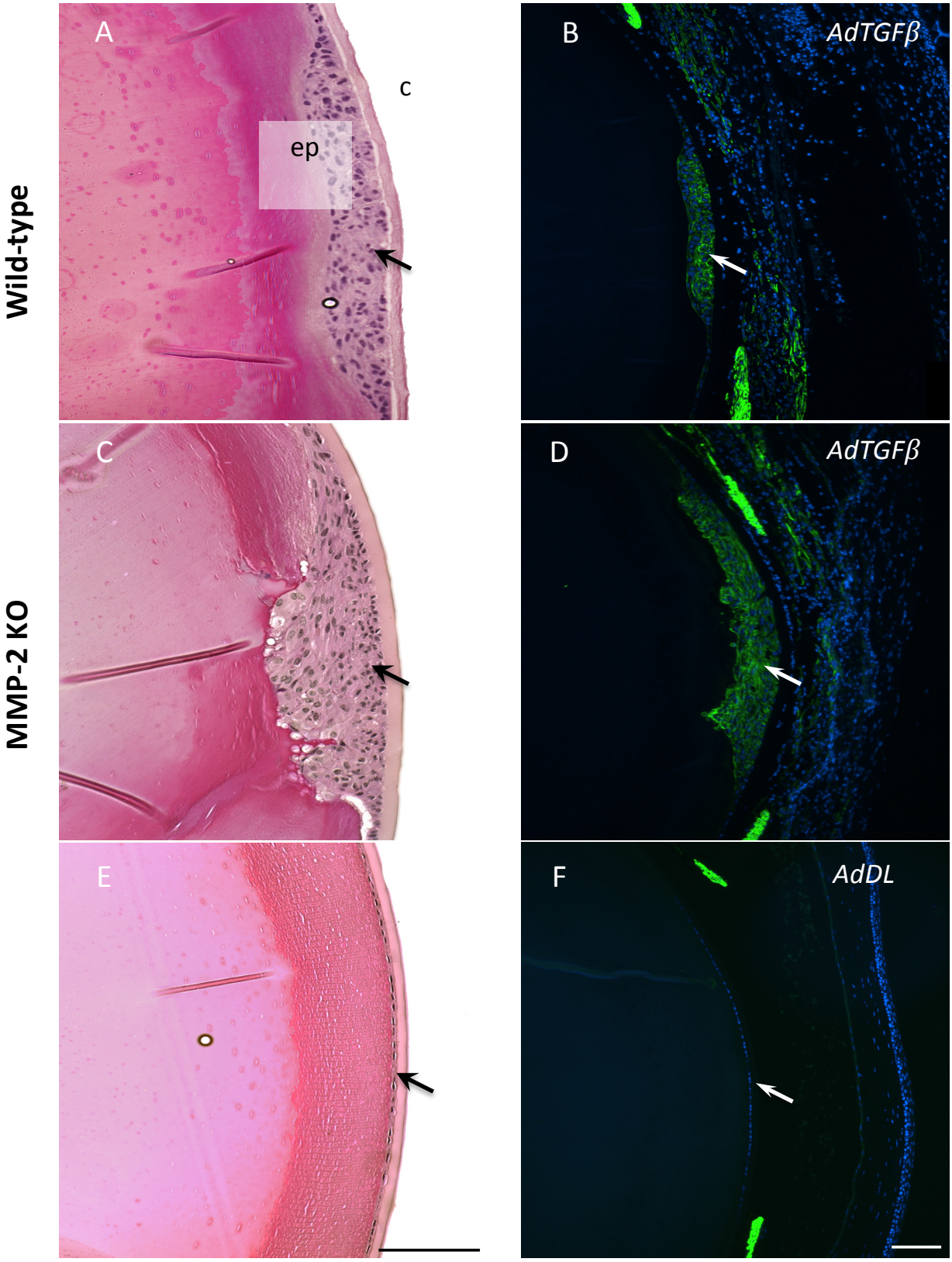

Wild-type
MMP-2 KO
A
c
ep
B
AdTGFβ
C
D
AdTGFβ
E
F
AdDL

Figure 7. Effects of AdTGFβ1 injection on lens epithelium after twelve days. Histological sections from mice injected with AdTGFβ1 (*A, B, C , D*) or AdDL (*E, F*). Sections were stained with H&E (*A, C, E*) or subjected to immunostaining for αSMA (*B, D, F*). Focal multilayering of LECs (*ep*) occurs in both WT and MMP-2 KO mice treated with AdTGFβ (*A, C, black arrows*) and in both cases is associated with induction of αSMA (*B, D, white arrows*). All mouse eyes injected with AdDL maintained a monolayer of LECs (*E, black arrow*) that did not express αSMA (*F, white arrow*). Note the presence of an intact lens capsule (*c*) in all lenses following adenoviral injection. In the immunostained images green stain is αSMA and the blue stain is DAPI. *c,* lens capsule. *ep,* lens epithelium. Scale bars = 100 μm.

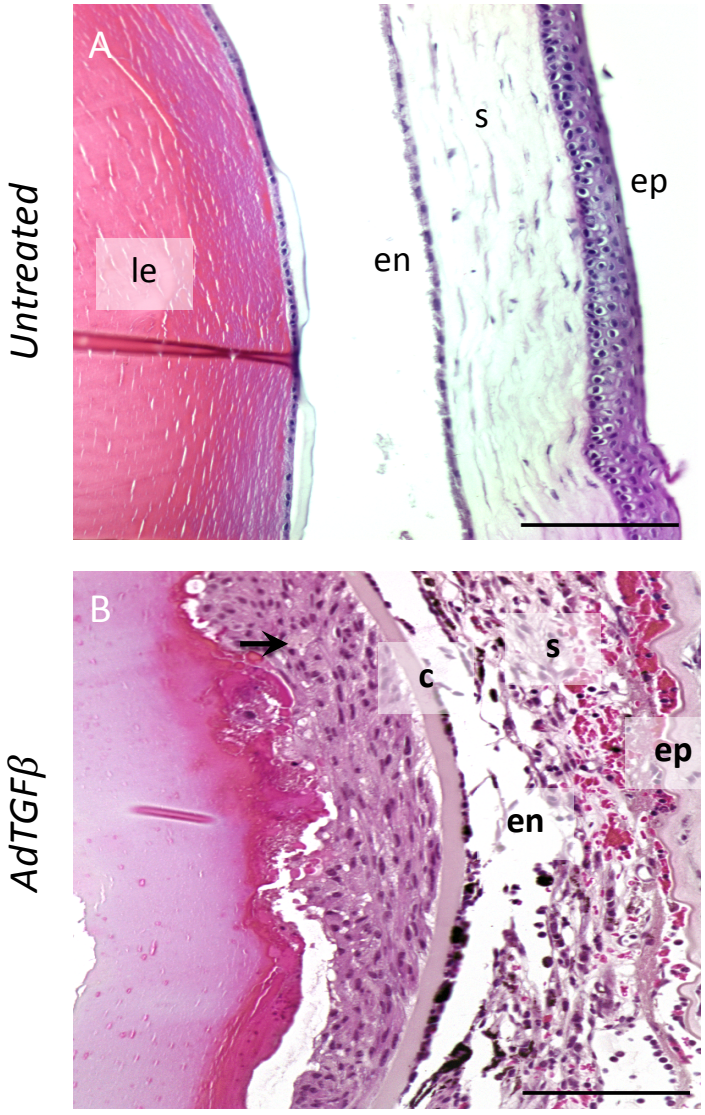


Figure 8. **Corneal disorganization in response to AdTGFβ1**. Histological sections of mouse eyes left untreated (*A*) or injected with AdTGFβ1 (*B*). AdTGFβ injection caused a thickening of the strom (*s*), disorganization of the corneal epithelium (*ep*), a scatter or loss of the continuous corneal endothelium (*en*) allowing for stromal cells to be in contact with the lens capsule (*c*). Note anterior subcapsular plaque formation (*B, black arrow*) in conjunction with corneal disorganization in response to TGFβ treatment. *c,* lens capsule. *en,* corneal endothelium. *ep,* corneal epithelium. *s,* corneal stroma. Scale bars = 100 μm.

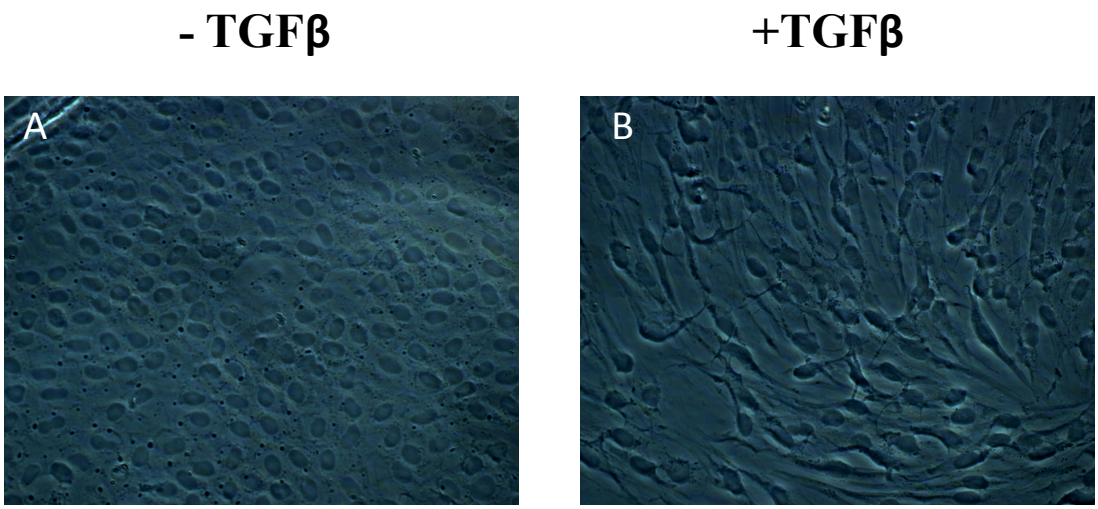


Figure 9. Phase contrast images depicting TGFβ-induced transformation in lens explants. Untreated cells (*A*) possess clear cell-cell contacts, and a cuboidal arrangement, while TGFβ treatment (*B*) leads to the loss of cell-cell adhesion, and cell elongation.

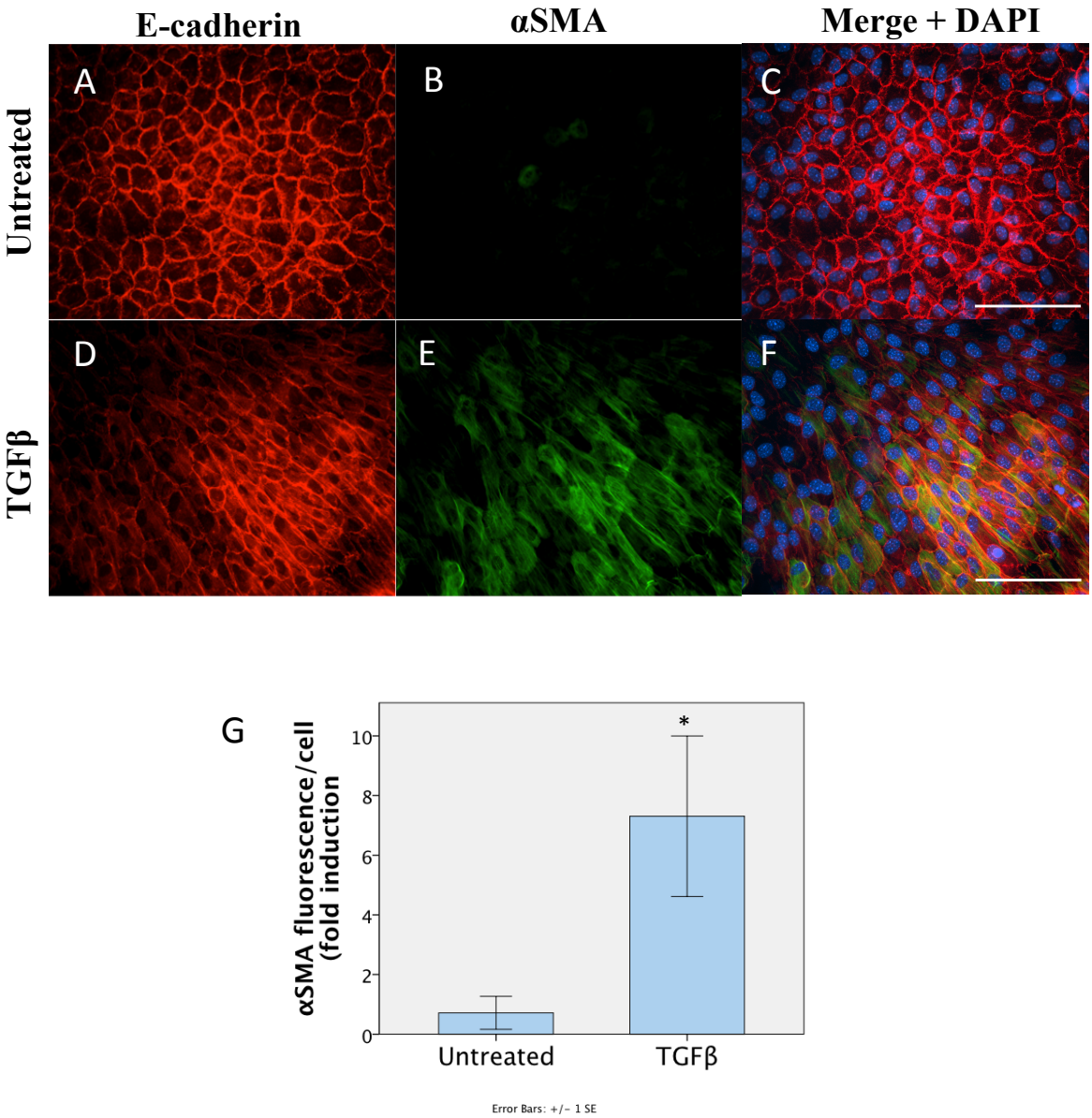


Figure 10. Effects of TGFβ treatment on WT mouse lens epithelial explants. Confluent lens explants when left untreated maintain a tightly packed, cuboidal, "cobblestone"-like appearance in a monolayer (n=7) with E-cadherin expressed at cellular junctions (*A*) and negligible αSMA expression (*B*). When LECs are treated with TGFβ (n=7) they express less E-cadherin at cellular junctions (*D*) and exhibit distinct αSMA expression (*E*). TGFβ leads to a significant (~7-fold) induction in αSMA compared to untreated WT lens explants (*G, *P*<0.05). Scalebar = 100μm

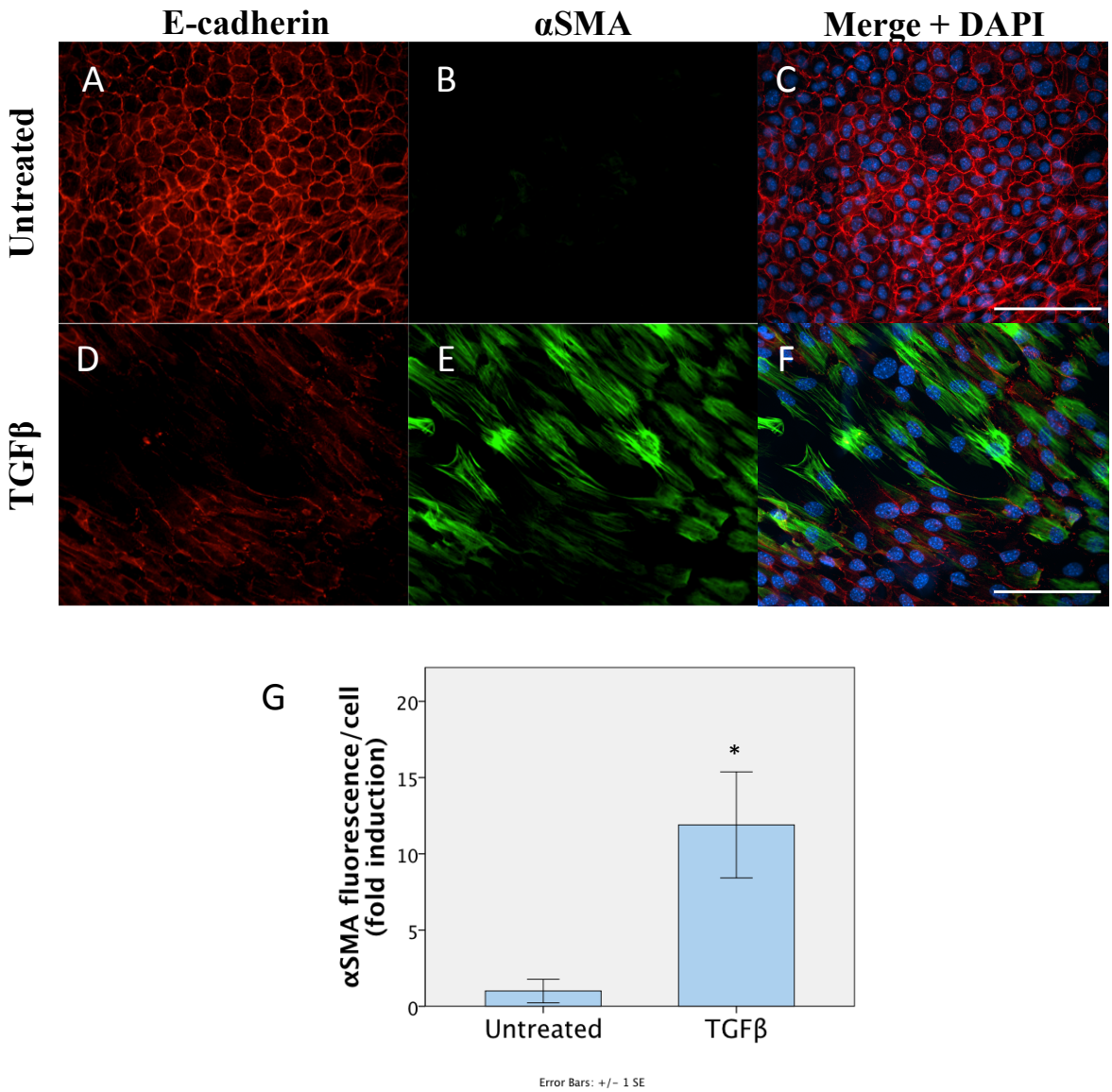


Figure 11. Effects of TGFβ treatment on MMP-2 KO mouse lens epithelial explants. MMP-2 KO explanted LECs, when left untreated (n=10), maintained a cuboidal arrangement with E-cadherin staining present at intact cellular junctions (A) and negligible αSMA expression (*B*) similar to WT LECs. In the absence of MMP-2, TGFβ induced a phenotypic transformation (n=7) with a distinct loss of E-cadherin (*D*) and marked expression of αSMA (*E*), similar to treated WT LECs. TGFβ leads to a significant (~12-fold) induction in αSMA compared to untreated MMP-2 KO lens explants (*G, *P* <0.05). Note the lack of overlap between E-cadherin and αSMA staining (*F*). Scalebar = 100μm

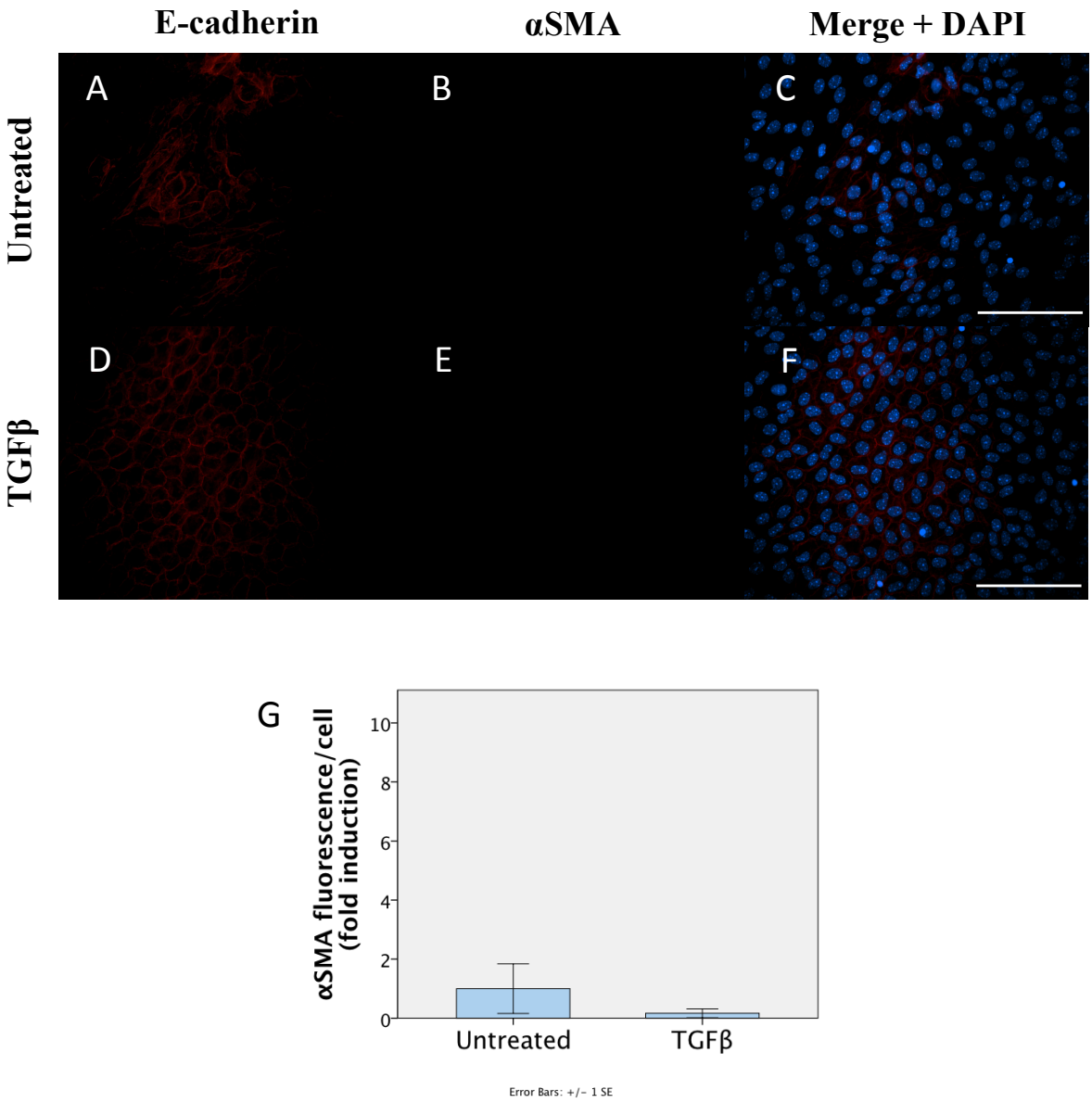


Figure 12. Effects of TGFβ treatment on MMP-9 KO mouse lens epithelial explants. MMP-9 KO LEC explants when left untreated (n=7) already expressed low levels of E-cadherin (*A*) with negligible αSMA expression (*B*). LECs treated with TGFβ in the absence of MMP-9 (n=5) exhibited low levels of E-cadherin at cellular junctions (*D*), and in contrast to TGFβ-treated WT and MMP-2 KO explants, did not express αSMA (*E*). TGFβ did not significantly induce αSMA expression when compared to untreated MMP-9 KO lens explants (*G*). Scalebar = 100μm

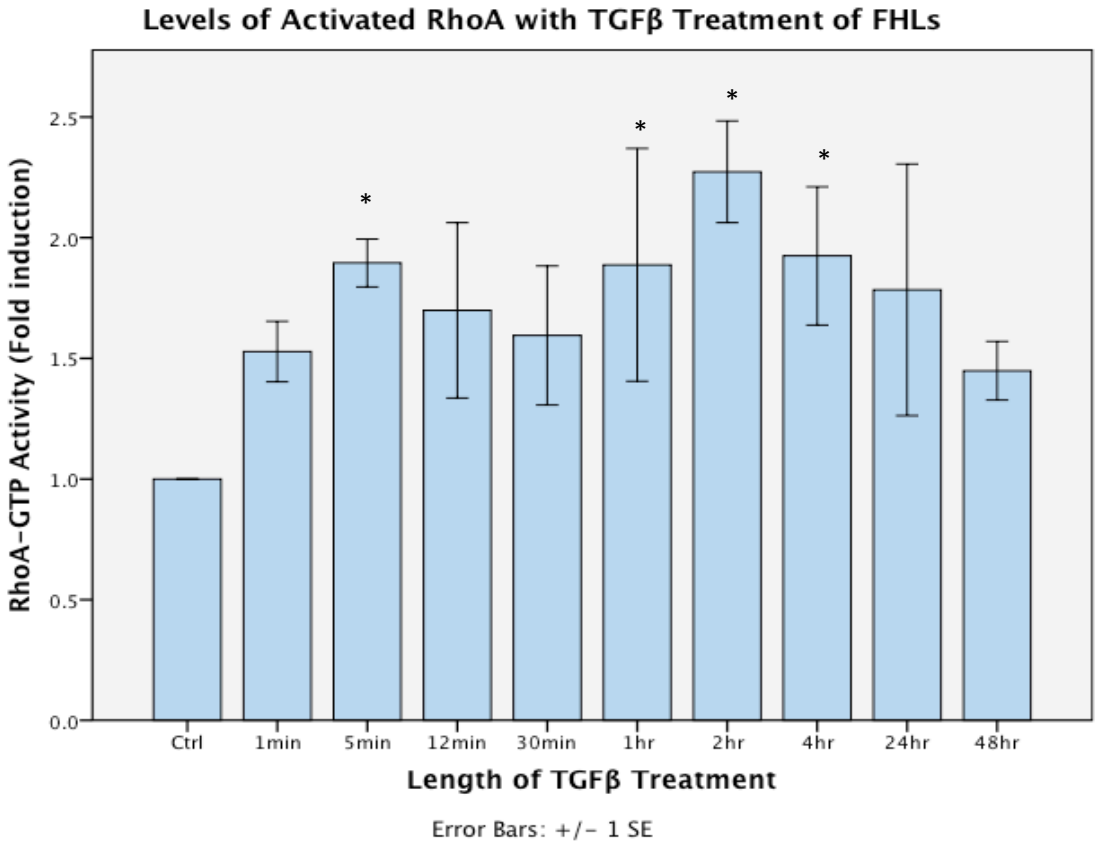


Figure 13. Activation of RhoA in response to TGFβ treatment across different time points. FHL 124 cells were exposed to TGFβ (4ng/ml) for the indicated time periods measuring level of RhoA activity using a RhoA activation assay. TGFβ significantly induces RhoA (GTP-bound form) after 5mins, 1hr, 2hrs, and 4hrs of treatment (n=3/time point; *P<0.05).

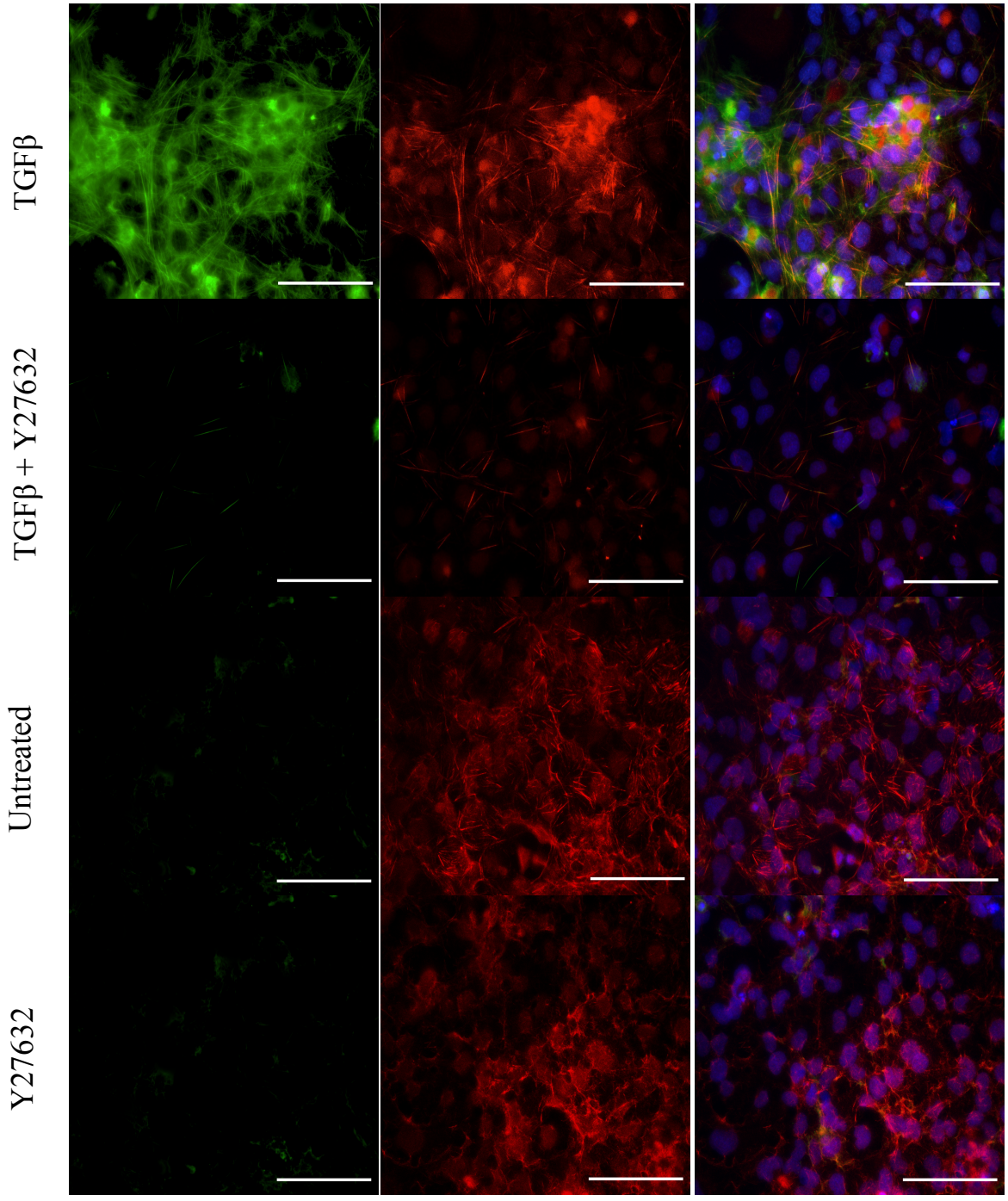


Figure 14. Effects of TGFβ treatment in the presence and absence of Y27632. FHL cells were treated with TGFβ (4ng/ml) with and without Y27632 (20μM). Cells were subsequently fixed and stained with αSMA and Phalloidin. The green stain is αSMA, red stain is F-actin, blue stain is DAPI. Scalebar = 100μm

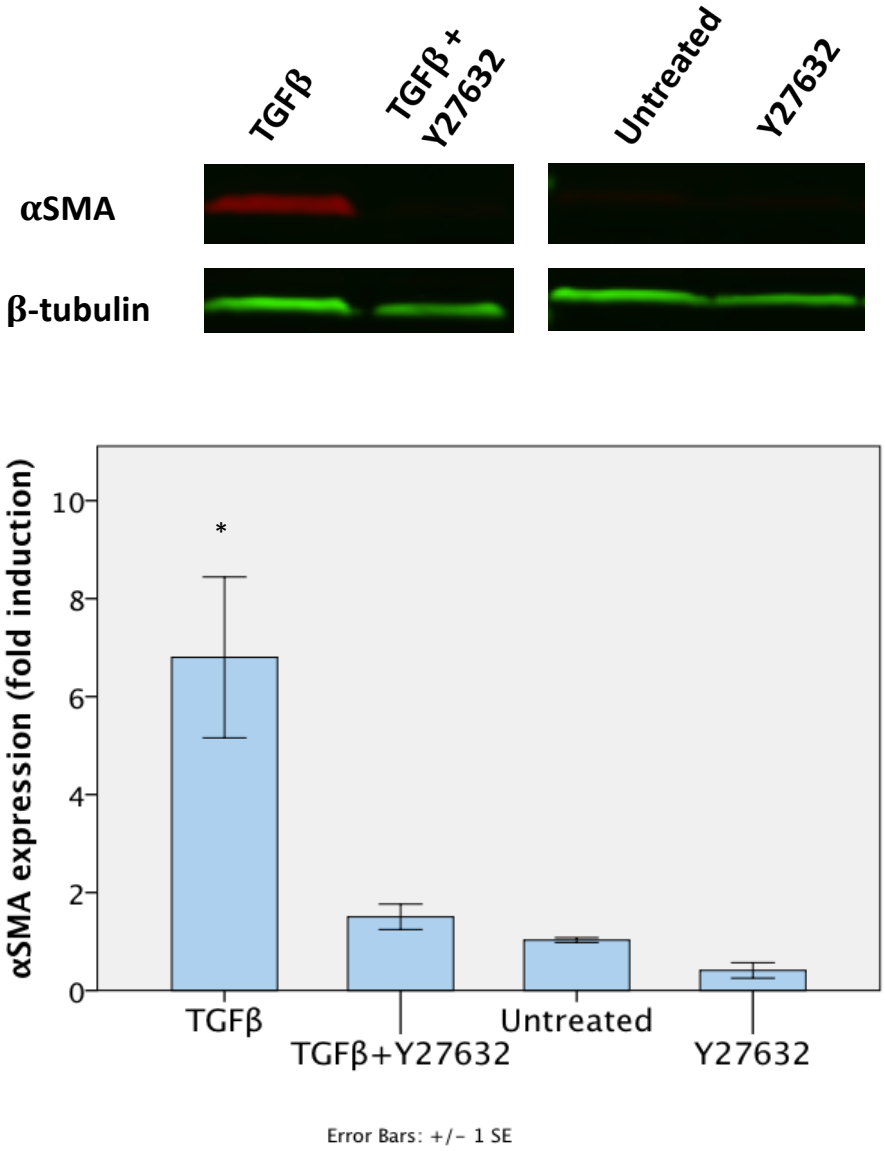


Figure 15. Quantifying αSMA in response to TGFβ in the presence and absence of Y27632. FHL cell lysates were collected after a 72 hour treatment period and a representative western blot and corresponding analysis is shown (n≥3 per treatment group). TGFβ (4ng/ml) significantly induced (~7-fold) αSMA expression compared to untreated FHLs. Co-treatment with TGFβ (4ng/ml) and Y27632 (20μM) prevented the induction of αSMA with expression not significantly differing from untreated FHLs. (*P<0.05)

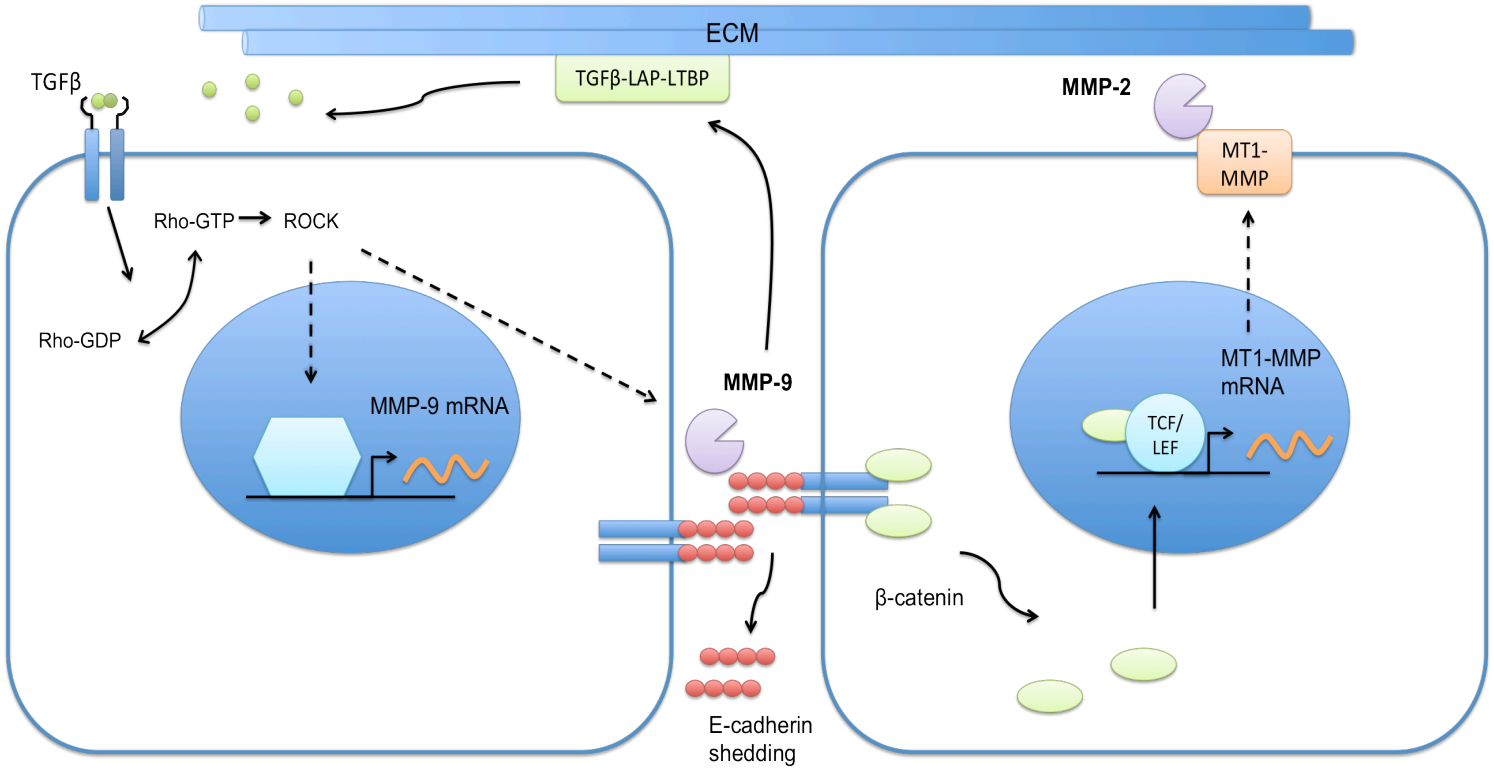


Figure 16. Proposed mechanism for TGFβ-induced MMP-2 and -9 induction. TGFβ activates RhoA/ROCK signaling which may lead to an induction in the proteolytic activity of MMP-9 in the extracellular space near the cell membrane. Here MMP-9 is in close proximity with E-cadherin at the cellular junctions and leads to the shedding of the ectodomain of E-cadherin. The loss of E-cadherin disrupts cell-cell contacts and facilitates the dissociation of β-catenin from the cytoplasmic region of E-cadherin. Once free in the cytoplasm, β-catenin translocates into the nucleus where it forms a complex with Tcf4/LEF leading to an increase in MT1-MMP mRNA. At the cell membrane MT1-MMP activates MMP-2. Beyond its effects on E-cadherin, MMP-9 is also able to activate further TGFβ by releasing it from its latency complex embedded in the ECM exacerbating TGFβ signaling and its induction of EMT.

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